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# 702. WINTER FEEDING OF DAIRY COWS

## V. A COMPARISON OF THE RELATIVE VALUE FOR MILK PRODUCTION OF ARTIFICIALLY DRIED COCKSFOOT AND A TIMOTHY/MEADOW-FESCUE MIXTURE GROWN BOTH IN SPRING AND IN AUTUMN

BY R. C. CAMPLING* AND W. HOLMES†

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It is generally accepted that the most important factor governing the feeding value of artificially dried grass is the stage of growth at which the grass is cut (1). There is some evidence, however, that different species of grass or different mixtures of grass species may be of different feeding value as judged by the volume of milk produced per acre (2,3) by the persistency of milk yield (4), and by differences in the output of meat per acre from cattle and sheep (5,6). Because of the increasing popularity of special purpose grass leys and the continued use of dried grass in feeding dairy cows, a comparison was made of the relative value for milk production of artificially dried grass from two contrasting types of ley. The opportunity was taken also to investigate further the relative value for milk production of spring- and autumn-grown dried grasses.

The grasses chosen for comparison were cocksfoot and a mixture of timothy and meadow-fescue. Cocksfoot was selected because, of all common grasses, it is the one whose value for milk production is most questioned (4). Cocksfoot, however, possesses several characteristics which make it a valuable grass for drying purposes. It is capable of high yields and is relatively highly efficient in utilizing fertilizer nitrogen in comparison with other common grasses (7). The timothy/meadow-fescue grass mixture, with which the cocksfoot was compared, was chosen because in recent years its use appears to have increased at the direct expense of cocksfoot leys (8) and unlike cocksfoot its palatability to stock is regarded as being very high.

### EXPERIMENTAL

#### *Treatments*

The cocksfoot and the timothy/meadow-fescue mixture were each cut and artificially dried, in both spring and autumn.

There were thus four grasses for comparison as follows: A, cocksfoot grown in the spring; B, cocksfoot grown in the autumn; C, timothy/meadow-fescue mixture grown in the spring; D, timothy/meadow-fescue mixture grown in the autumn. The two fields in which the cocksfoot and timothy/meadow-fescue mixtures were grown were adjacent and of the same soil type and had received almost identical management during the 8 years preceding the present experiment. Both the cocksfoot ley and the timothy/meadow-fescue ley received similar generous fertilizer applications (60 lb. N and 60 lb. $K_2O$/acre) 4–5 weeks before each cut. The four grasses were cut when about 10 in. high and in the

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pre-flowering stage of growth. After it was cut the grass was wilted in the field for 1–12 hr. and then dried in a low-temperature tray-type drier (I.C.I. Mark 3). The grass was baled and stored indoors. The dates of cutting of the grasses are given in Table 1. The dried

Table 1. *The dates of cutting the grasses*

| Treatment | Date of cutting |
|---|---|
| A: spring cocksfoot | 24 May 1955 |
| B: autumn cocksfoot | 20 September 1955 |
| C: spring timothy/meadow-fescue | 31 May 1955 |
| D: autumn timothy/meadow-fescue | 26 September 1955 |

grass fed as treatment A consisted of cocksfoot (*Dactylis glomerata*) with some 15% of weed grasses, mainly meadow grasses (*Poa* spp.). The autumn-grown grass (treatment B) was mainly cocksfoot with a small quantity of red fescue (*Festuca rubra*). The grass of treatment C consisted of approximately 50% timothy (*Phleum pratense*), 40% meadow-fescue (*Festuca pratensis*) and 10% weed grass (*Poa* and *Festuca* spp.). The autumn-grown timothy/meadow-fescue mixture (treatment D) contained slightly more meadow-fescue than timothy and a very small proportion of weed grasses.

### *Experimental design*

Eight autumn-calving Ayrshire cows were grouped into two balanced 4 × 4 Latin squares on the basis of their calving date (9). The first square contained cows which had calved about 12 weeks, and the second contained cows which had calved about 7 weeks before the experiment began. Within each square the cows were allocated at random to the four treatment sequences. The cows received a 14-day preliminary period on standard weighed rations before the first experimental period began. The experimental periods were each of 3 weeks' duration. The changeover from one treatment to another was made abruptly, but for the purposes of records a 36 hr. interval was allowed before milk production was credited to any particular treatment. During the first period one cow had to be removed from the experiment because of mastitis, and another cow was substituted. The missing plot technique of Yates (10) was used to complete the results for the purposes of statistical analysis.

### *Feeding and management*

The daily experimental ration offered to each cow consisted of 17 lb. of one of the four dried grasses under comparison, and 28 lb. of fodder beet. A concentrate mixture (starch equivalent 66, digestible crude protein 12) was fed to each cow at the rate of 5 lb. per gallon of milk produced after the first 1½ gallons per day. The total amount of concentrate fed to each cow daily was adjusted at the beginning of each period to allow for declining yield with advancing lactation using a method based on the equalized feeding principle of Lucas (11). The quantity of dried grass and fodder beet offered was constant throughout the experiment. All foods offered to each cow were weighed daily and a record of all food refusals was kept on 3 consecutive days each week. Samples of dried grass, fodder beet and concentrates were taken weekly for dry-matter determination and chemical analysis. The crude fibre content of the grasses was determined by the method of Whitehouse, Zarrow & Shay (12). The content of water-soluble carbohydrates of each of the four grasses was determined on composite samples of the grasses by the method of

Waite & Boyd (13) on a hydrolysed, cold water extract of the grasses. The approximate nutritive values of the foods in terms of starch equivalent (S.E.) and protein equivalent (P.E.) were calculated from values published by Woodman (14) for concentrates and from values by Castle, Foot & Rowland (15) and Bailey, Castle & Foot (16) for fodder beet (S.E. 50; P.E. 3·5 of dry matter). For the dried grasses the prediction equations of Watson & Horton (17) were used: S.E. $= 0{\cdot}8692x + 38{\cdot}145$; P.E. $= 0{\cdot}9642x - 5{\cdot}230$; where $x$ = the percentage of crude protein in the dry matter. A composite sample of milk was taken from each cow on one day each week for the determination of butterfat content by the Gerber test (B.S.S. no. 696, 1955) and the solids-not-fat (S.N.F.) content by a density method (B.S.S. no. 734, 1955). The cows were weighed at 3 p.m. on 3 consecutive days each week. During the last week of each period an index of the palatability of each dried grass was obtained by measuring the amount of dried grass consumed per unit of time. This was done at the 11.30 a.m. feed when the cows were offered 4 lb. of dried grass. The cows were kept in a cowshed of conventional design throughout the experiment and water was available to them at all times.

## RESULTS

### *Chemical composition of the grasses*

The chemical composition of the dried grasses is summarized in Table 2. The contents of crude protein in the spring- and autumn-grown cocksfoot were similar to that of the spring-grown timothy/meadow-fescue mixture, but the autumn-grown timothy/meadow-fescue had a higher content of crude protein than the other three grasses. Both the

Table 2. *The chemical composition of the dried grasses*

| Treatment | Dry matter (%) | As percentage of dry matter: Crude protein | Crude fibre | Total ash | Silica-free ash | Water-soluble carbohydrates |
|---|---|---|---|---|---|---|
| A: spring cocksfoot | 88·0 | 14·8 | 21·5 | 8·2 | 6·6 | 15·1 |
| B: autumn cocksfoot | 88·8 | 14·9 | 27·9 | 12·3 | 8·8 | 5·6 |
| C: spring timothy/meadow-fescue | 88·4 | 14·2 | 23·5 | 6·6 | 5·6 | 13·6 |
| D: autumn timothy/meadow-fescue | 88·0 | 19·9 | 26·3 | 14·6 | 7·9 | 4·7 |

grasses grown in the autumn were similar in crude fibre content and were slightly higher in crude fibre than the spring grown grasses. The autumn-grown grasses were also higher in ash and silica contents than the spring grasses. The content of soluble carbohydrate in the grasses grown in the spring was approximately three times as great as that found in the autumn-grown grasses. There was little difference in the content of soluble carbohydrates between the cocksfoot and the timothy/meadow-fescue mixture.

### *Food consumption*

Of the foods offered to the cows practically all were consumed. The amounts of dry matter eaten under each treatment are given in Table 3. Small residues of dried grass occurred occasionally, and it was observed that this happened only when one or other of the autumn-grown grasses was fed. On the whole, these refusals were small and none of the differences between the intakes of the four dried grasses was statistically significant. There were no significant differences between the amounts of concentrates and fodder

Table 3. *The average feed consumption*

(Dry matter lb./cow/day.)

| Treatment | Grass | Concentrates | Fodder beet |
|---|---|---|---|
| A: spring cocksfoot | 15·0 | 10·9 | 5·5 |
| B: autumn cocksfoot | 14·8 | 10·9 | 5·5 |
| C: spring timothy/meadow-fescue | 15·0 | 10·9 | 5·5 |
| D: autumn timothy/meadow-fescue | 14·9 | 10·8 | 5·5 |
| Standard error of difference between two means | ±0·12 | ±0·07 | — |

Table 4. *The estimated intakes of starch equivalent and protein equivalent on each treatment*

(lb./cow/day.)

| | Grass | | Concentrates | | Fodder beet | | Total | |
|---|---|---|---|---|---|---|---|---|
| Treatment | S.E. | P.E. | S.E. | P.E. | S.E. | P.E. | S.E. | P.E. |
| A: spring cocksfoot | 7·7 | 1·3 | 7·2 | 1·3 | 2·8 | 0·2 | 17·7 | 2·8 |
| B: autumn cocksfoot | 7·6 | 1·4 | 7·2 | 1·3 | 2·8 | 0·2 | 17·6 | 2·9 |
| C: spring timothy/meadow-fescue | 7·7 | 1·3 | 7·2 | 1·3 | 2·8 | 0·2 | 17·7 | 2·8 |
| D: autumn timothy/meadow-fescue | 8·3 | 2·1 | 7·2 | 1·3 | 2·8 | 0·2 | 18·3 | 3·6 |

beet consumed with each treatment. The average daily intake of dry matter for each cow was equivalent to 2·9% of the live weight, and of this dry-matter intake 48% was dried grass. In terms of starch and protein equivalent intakes (Table 4) there was little difference between the total intakes with treatments A, B and C, but there was a slightly higher intake of starch and protein equivalent with the autumn timothy/meadow-fescue. The intakes of starch equivalent with all four treatments were about optimal for economic milk production (18,19). Although the intakes of protein with treatments A, B and C were a little lower than that of the Woodman standard (14) this probably did not result in underfeeding of protein since the Woodman standard for protein is regarded as slightly too high (20).

Table 5. *Index of palatability of the dried grasses*

| Treatment | Rate of eating (min./lb.) of grass dry matter |
|---|---|
| A: spring cocksfoot | 3·6 |
| B: autumn cocksfoot | 7·1 |
| C: spring timothy/meadow-fescue | 4·1 |
| D: autumn timothy/meadow-fescue | 6·0 |
| Standard error of difference between two means | ±0·81 |

The index of the palatability of the four dried grasses obtained by recording the time taken by each cow to consume the dried grass is given in Table 5. It was found that the spring dried grasses were both eaten more quickly than the autumn grasses. Statistical analysis of the results showed that this difference was highly significant ($P < 0{\cdot}01$) but there was no statistically significant difference between species.

*Animal production*

The animal production results are summarized in Table 6. These values are derived from results collected over the whole of each 3-week period. An analysis of the results of the last 2 weeks of each period was made so as to eliminate the introductory week, but the results were similar to those of the 3-week period.

Table 6. *Animal production during the experiment*

| Treatment | Mean daily milk yield (lb./cow/day) | Milk composition | | Mean live weight (lb./cow) |
|---|---|---|---|---|
| | | Mean fat % | Mean S.N.F. % | |
| A: spring cocksfoot | 36·6 | 4·2 | 8·7 | 1105 |
| B: autumn cocksfoot | 35·0 | 4·3 | 8·5 | 1092 |
| C: spring timothy/meadow-fescue | 36·1 | 4·3 | 8·6 | 1097 |
| D: autumn timothy/meadow-fescue | 35·6 | 4·2 | 8·5 | 1064 |
| Standard error of difference between two means | ±0·75 | ±0·16 | ±0·05 | ±6·5 |

*Milk yield*

With the exception of one cow which contracted mastitis and which had to be replaced, satisfactory milk yields were maintained throughout the experiment, and the average yield was 35·8 lb. per cow per day. Despite the fitting of a missing value the coefficient of variation of the milk-yield results was only 4·2% and this compares favourably with that obtained in other experiments conducted with the Hannah Institute herd when the same type of design was used (21). No difference in mean milk yield was found between cocksfoot and the timothy/meadow-fescue mixture. With both grasses there was a slightly higher mean yield with the spring grasses than with the autumn-grown grasses, but the difference was small and statistically non-significant. There were no significant residual effects of one treatment upon another.

*Milk composition*

The average composition of the milk is shown for each treatment in Table 6. The fat content of the milk was satisfactory and averaged 4·3%. There were only very small differences in fat content between the treatments and these were not statistically significant. The mean solids-not-fat content over all treatments was somewhat low (8·6%) and with both cocksfoot and timothy/meadow-fescue the spring-grown grass produced a higher mean solids-not-fat content than did the autumn-grown grass. This effect was significant with cocksfoot ($P < 0{\cdot}05$) but not with the timothy/meadow-fescue.

*Live weight*

The mean live weights on each treatment ranged from 1064 to 1105 lb., and a highly significant depression in live weight was found with the autumn timothy/meadow-fescue mixture ($P < 0{\cdot}001$). There were no statistically significant differences between the live weights on the other three grasses, although the feeding of the autumn cocksfoot led to a lower live weight than the spring cocksfoot. The changes in live weight took place within the first week after the change of treatment, and the differences between the treatments did not change materially after the first week of each period. The fact that the substantial average loss of 34 lb. per cow occurred within 1 week of changing to the autumn-produced timothy/meadow-fescue from the other grasses suggests that the main reason for the depression in live weight was a change in the amount of the contents of the rumen and intestines and not to a change in body tissue.

## DISCUSSION

No differences in value for milk production between artificially dried cocksfoot and a timothy/meadow-fescue mixture cut at similar stages of growth were found. This result confirms the findings obtained in Germany where hays made from the more commonly sown grasses of agricultural importance were observed to be of similar milk producing value (22). Differences between grass species and their effects on milk production have been reported only from grazing studies (2,3,4), but this does not necessarily imply any differences in feeding value per unit of dry matter. Differences in production per acre, seasonality of growth, or palatability to the grazing animal are most probably responsible.

No statistically significant differences in milk yield were found between the feeding of spring- and autumn-produced dried grass, but despite this it is interesting to note that feeding spring grass resulted in a slightly higher milk yield. This result is very similar to that reported by Holmes (23) in earlier work at the Hannah Institute when a 3–5% increase in milk yield was found with artificially dried spring grass, compared with autumn grass, although again the difference was statistically non-significant. The results of these experiments, together with those of Morris, Wright & Fowler (24) and of Dijkstra (25), suggest that if artificially dried spring grass has any special virtue for milk production its importance is very small. In fact, the superiority of spring grass over autumn grass has been reported only from observations made under grazing conditions (26,4). It may be that autumn grass grazed *in situ* is not eaten in adequate amounts because its palatability is reduced by some environmental factor such as heavy dew or soil splash (27) or by the presence of fungal infections.

The reason for the small increase in the solids-not-fat content of the milk resulting from the feeding of the spring grass is not known. The content of soluble carbohydrates in the spring grasses was very much higher than that of the autumn grasses, and Waite, White & Robertson (28) have suggested that the higher proportion of water-soluble carbohydrate normally found in spring grass may result in an improvement in the plane of nutrition of the cow and thus in an increase in the protein content of the milk. Another explanation of the rise in the solids-not-fat content of milk with spring herbage may be in its oestrogen content (29).

In general, the results imply that the effects of season of growth and species on the feeding value of artificially dried grass may be virtually ignored and that the relative value for artificial drying of a grass species may be judged primarily on agronomic characteristics such as the efficiency of nitrogen utilization or seasonality of growth.

## SUMMARY

1. An experiment was conducted to compare the relative value for winter milk production of artificially dried cocksfoot and a mixture of timothy and meadow-fescue each cut in the spring and autumn.

2. A double balanced $4 \times 4$ Latin square design with eight Ayrshire cows and four periods each of 3 weeks' duration was used.

3. The dried grasses were fed in equal amounts (17 lb./cow/day) and equal quantities of each grass were consumed in terms of dry matter. Approximately half the total dry matter intake was grass.

4. No differences were detected between the cocksfoot and the timothy/meadow-fescue mixtures in their value for milk production.

5. With both cocksfoot and timothy/meadow-fescue the spring-grown grass gave slightly higher milk yields than the autumn-grown grasses but the differences were not statistically significant.

6. Feeding the spring-grown dried grasses led to a slightly higher solids-not-fat content in the milk than when the autumn-grown grasses were fed.

7. The feeding of the autumn grown timothy/meadow-fescue grass mixture was associated with a significant loss of live weight in the cows. It was suggested that this loss of weight was due primarily to changes in the amount of the contents of the rumen and intestines.

The authors thank Dr R. Waite and Mr N. H. Strachan for chemical analysis of the dried grasses and other feedingstuffs. One of us (R. C. C.) is indebted to the Ministry of Agriculture, Fisheries and Food for the award of a scholarship.

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# 703. CINERADIOGRAPHIC OBSERVATIONS ON MACHINE MILKING

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(With 3 Figures)

Milk is obtained from the teat during machine milking by the creation of a partial vacuum; the rate of flow is influenced by many factors including changes in the vacuum, the pulsation rate or ratio, alterations in the design or construction of the teat cup liner. Recent research relating to these problems has been reviewed by Dodd & Clough (1).

The rate of milk flow or machine milking rate is usually expressed as the mean rate of flow from one or more teats over a specified period of milking. In this way it is possible to show whether a change in the milking machine affects milk flow but not how the change is brought about; for example, it is possible to demonstrate that an increase in pulsation rate and teat liner tension causes much faster milking but the reason remains obscure. In order to discover why such changes in milking rate occur it is necessary to measure the proportion of time during which milk flows in each pulsation cycle and the rate of flow of milk during the flow period. In this investigation we have studied some aspects of this problem with the aid of a portable X-ray cine-camera (2).

## METHOD

Three cows accustomed to machine milking were milked in a two-level stall designed so that the movement of the cow could be restricted. The left front teat of each cow was turned up against the udder and held in that position with adhesive tape to allow an unrestricted view of the right front teat. The right front quarter was then milked until most of the milk was removed. The teat cup assembly was then removed from the teat and a warm (100° F.) sterile suspension of barium sulphate (Micropaque: Messrs Damancy) was infused into the udder through the teat orifice with an enema syringe. The aim was to achieve a 25% suspension of barium sulphate in the udder contents so that there was sufficient opacity for the cineradiography.

The teat cup assembly consisted of a normal extruded teat cup liner (Alfa Laval 20003B) fitted into a glass teat cup of standard size. When a liner is caused to pulsate the collapse is always in the same plane: in this experiment the liner was applied to the teat so that the plane of the collapse was parallel to the X-ray beam. The milking machine vacuum under different conditions was measured with a mercury manometer, the pulsation ratios had been previously determined by a cine-camera technique (3), and changes in liner tensions were measured with a spring balance (4). The X-ray camera was operated at 50 frames/sec.: the exposure time for each frame was $\frac{1}{100}$ sec.

Twenty film sequences were taken, each of 5 sec. duration. Observations were made upon the movements of the teat cup liner, the size of the teat sinus, the opening and closing of the teat orifice and the proportion of time during which the milk was flowing in each cycle, though not the volume flowing. The machine was adjusted to provide pulsation rates ranging from 40 to 240 cyc./min., pulsation ratios of 1 to 1 and 3 to 1 and teat cup liner tensions of 5–25 lb.

## RESULTS

The changes shown in a typical cycle of movements, when the pulsation rate was 40 cyc./min., the pulsation ratio one to one and a teat cup liner tension of 25 lb., are shown in Fig. 1. When the liner (below the end of the teat) was fully collapsed the teat was narrowed, the degree of narrowing being greater at the tip than at the base. The tip of the teat did

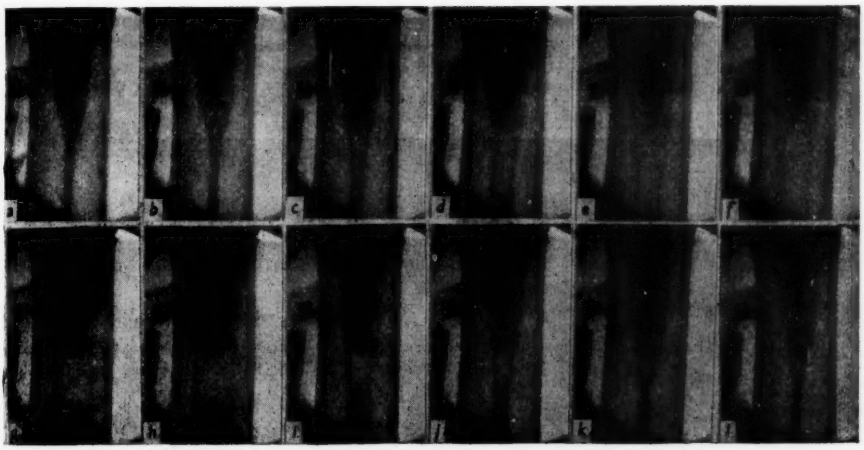

Fig. 1. A series of prints of consecutive frames from a cineradiographic film taken at 50 frames/sec. of the teat and teat cup assembly. The appearances are shown diagrammatically in Fig. 2. Pulsation rate 40 cyc./min., pulsation ratio 1:1 and liner tension 25 lb. (*a*) Stage of maximum collapse of the teat and liner. (*b*) Liner beginning to expand; teat sinus increased in volume. (*c*) Further expansion of liner and teat; milk entering streak-canal. (*d–f*) Further expansion of liner and teat. A jet of milk impinges on the side wall of the liner. (*g, h*) Maximum expansion of the liner; milk flowing; barrel of the liner partly filled with milk. The appearances remained unchanged for the next 30 frames. (*i–l*) Liner collapsing: milk flow interrupted at *i*.

not completely fill the space above the completely collapsed liner; this space is shown on the prints as a translucent zone. There was a little narrowing of the base of the teat at the level of the metal ring in the top of the liner. The liner enclosing the teat gradually narrowed from the metal ring above to where its walls were in apposition. The opacified milk filled the teat sinus; there was no milk in the *ductus papillares* or streak-canal (Figs. 1*a*, 2).

Radiographically the first change seen was an increase in the width of that part of the liner enclosing the teat and an increase in the size of the teat sinus (Fig. 1*b*). The teat cup liner then opened (Fig. 1*c–f*): the streak-canal began to open (Fig. 1*c*) and milk flowed from the teat (Fig. 1*d*). Milk continued to flow as the liner became fully opened and the teat sinus became fully expanded (Fig. 1*g*). Thus, milk flow began within one

twenty-fifth of a second of the liner beginning to open (Fig. 1*b–d*). Maximum expansion of the liner and teat was maintained for three-fifths of a second (Fig. 1*g–h*) and then the liner began to collapse (Fig. 1*c*); flow ceased within one-fiftieth to one twenty-fifth of a second. Complete collapse of the liner occurred within two twenty-fifths of a second (Fig. 1*c–e*). The barrel of the liner partly filled during the flow period of each pulsation cycle.

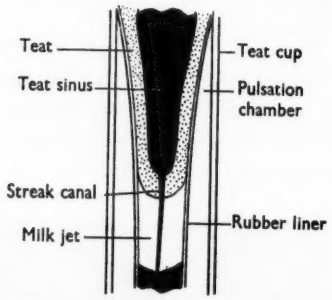


Fig. 2. Diagram to illustrate the structure radiographed.

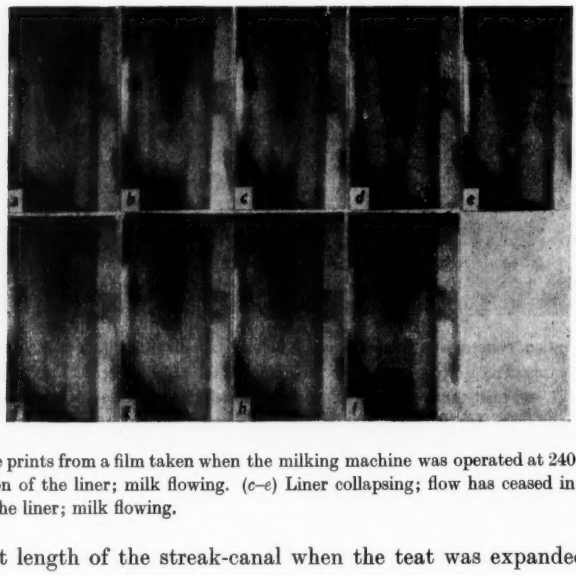

Fig. 3. Consecutive prints from a film taken when the milking machine was operated at 240 cycles. (*a, b*) Maximum expansion of the liner; milk flowing. (*c–e*) Liner collapsing; flow has ceased in *d* and *e*. (*f–i*) Re-expansion of the liner; milk flowing.

The apparent length of the streak-canal when the teat was expanded was less than when the teat was collapsed. When the udder was nearly empty the constriction in the teat in the region of the top of the liner was more pronounced: when the liner was collapsed the lumen was occluded at this level. When the liner was expanded the teat sinus was not as distended as when the udder was full, and the walls of the teat sinus appeared thickened.

When the milking machine was operated at a pulsation rate of 240 cyc./min. the liner walls were almost continuously moving; milk flow was only interrupted for about one twenty-fifth of a second (Fig. 3*d, e*). As with the slower rate of pulsation (Fig. 1), it was observed that when the collapsed liner began to expand there was an increase in the size of the teat sinus, and this occurred before milk flow was established.

The effect of changes in the properties of the milking machine or liner movement and the proportion of each cycle in which milk flows from the teat are given in Table 1, together with the results obtained by the direct cine-camera technique.

The milk subsequently obtained from a quarter infused with the barium suspension contained clots for several milkings, and during this period the udder was slightly swollen and tender. This reaction was reduced by washing out the quarter with sterile saline immediately the radiography was completed.

Table 1. *Measurement of milk flow with various pulsation rates and ratios and at liner tensions of* 5 *and* 25 *lb*

| | | | A. Measured by the direct cinematographic technique (3) | | | B. Measured cineradiographically | |
|---|---|---|---|---|---|---|---|
| | | | Pulsation | | | | |
| Exp. no. | Vacuum (in. Hg) | Liner tension (lb.) | Rate (cyc./min.) | Ratio | % open* | % open | % milk flow† |
| 1 | 15 | 25 | 40 | 1:1 | 50 | 53 | 55 |
| 2 | 15 | 5 | 40 | 1:1 | 50 | 53 | 55 |
| 3 | 15 | 25 | 40 | 3:1 | 75 | 77 | 81 |
| 4 | 15 | 25 | 160 | 1:1 | 50 | 63 | 74 |
| 5 | 15 | 25 | 160 | 3:1 | 75 | 89 | 89 |
| 6 | 15 | 25 | 240 | 0·75:1 | 43 | 66 | 75 |

* The proportion of each cycle that the liner is more than half open.

† The proportion of each cycle that milk is flowing from the teat.

## DISCUSSION

A radiographic study of the effects of mechanical milking and machine vacuum on the teat structure of an excised bovine mammary gland preparation has been published by Pier, Schalm & Hage (5). They took a series of still radiographs with an exposure of one-fortieth of a second during the milking cycle and distinguished milking (liner expanded) and resting (liner collapsed) phases. They postulated that the development of cineradiography would provide a better method for studying the sequel of changes. Our results on the living animal have confirmed their findings, the only difference being that when the udder was full there was not so great a degree of narrowing of the base of the teat sinus in our experiments; this may be due to the use of a different type of teat cup liner assembly or to a difference in the behaviour of the udder of the living animal as compared with the excised udder preparation. The radiographic appearances of the changes in the form of the teat sinus during the milking cycle differ from those observed in the cycle of movement in natural feeding (6) and from the changes in the form of a rubber teat when an animal is taking milk from a bottle (7).

Cineradiography at 50 frames/sec. provides a method of timing the sequence of events within a maximum error of one frame (one-fiftieth of a second); thus the results obtained by this method may be correlated with those obtained by other methods, including direct cinematography (as in this investigation) and pressure recordings. An advantage of cineradiography over direct cinematography is that one can determine when milk is flowing using standard rubber liners without the necessity for using transparent liners with different physical properties.

In this investigation the results obtained by cineradiography have been compared with the results obtained by the direct cinematographic method (3) by estimating the

proportion of the cycle in which the liner passed from half expanded through fully expanded to the half collapsed position. The cineradiographs show that milk flow stopped when the collapsing liner was about one half the normal width below the teat and restarted as soon as the liner began to open. In the direct cinematographic method (3) it was assumed that milk was flowing when the teat liner was more than half open. The results of the comparison of the two methods given in Table 1 show that the pulsation ratio as determined by cineradiography is slightly wider; this is probably due to the fact that when the teat is in the liner the collapse of the liner is resisted and is only complete below the tip of the teat, whereas in Clough & Dodd's (3) experiment a rubber bung was placed in the neck of the liner instead of the cow's teat. The difference between the two methods is small at normal pulsation rates but is larger with faster pulsations. It is considered that the direct cinematographic method has advantages as a routine test. Cineradiography also shows that until the udder is nearly empty there is no evidence that the flow of milk from the udder to the teat sinus is limited.

From Table 1 it can be seen that at a pulsation rate of 40 cyc./min. and ratios of either 1:1 or 3:1 (Exps. 1–3) milk flows for about 5% longer time in each cycle than estimated when it is assumed that milk is only flowing when the liner is more than half open; this is presumably because milk flows almost as soon as the liner begins to open. These results (Exps. 1 and 2) do not show that an alteration in liner tension affects the movements of the liner walls; we have other evidence which indicates that under certain conditions not employed in this investigation change in tension can affect liner movements sufficiently to account for an increase in the milking rate (8).

At pulsation rates of 160 and 240 cyc./min. (Exps. 4–6) the proportion of each cycle during which milk is flowing is considerably greater than the proportion of each cycle during which the liner is more than half open. This may be because the liner at the high pulsations is moving for over a third of each cycle as compared with about one-tenth of each cycle at 40 cyc./min.

SUMMARY

Machine milking has been studied in three cows by cineradiography at 50 frames/sec. The findings of Pier *et al.* (5) have been confirmed.

The effects of variation in the pulsation rate, ratio and liner tension were investigated and the results compared with those obtained by other methods. The proportion of each milking cycle during which milk flows has been determined.

We are grateful to Mr M. S. Tuckey for his assistance.

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# 704. THE PROPERTIES OF NEW ZEALAND BUTTERS AND BUTTERFATS

## V. MILKS AND BUTTERFATS FROM MONOZYGOTIC TWIN COWS

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(With 16 Figures)

Monozygotic twin cows have in recent years been used both overseas and in New Zealand in investigations on production and composition of milk. Hancock(1,2,3) discussed their advantages for this purpose and gave a table of 'twin efficiency values' for a number of milk composition characteristics. Patchell(4), more recently, has presented a table of 'twin efficiency values' covering a wider range of characteristics.

Since births of monozygous twins are relatively infrequent the twin sets are usually collected wherever they are obtainable, irrespective of the breed or productivity of their parents but not including pure beef breeds. The between-set differences, i.e. the variation between the means of the twin sets, are taken to be representative of differences that could occur between cows in a random sample of the cow population, except in so far as there is some selection, from among the sets of twins available, of those sets which appear most likely to be suitable for the experimental purposes in view.

For investigations on causes of variation in milk composition and in butterfat characteristics it has usually been found difficult, because of the extent of the cow-feeding and the analytical facilities required, to work with sufficient randomly selected unrelated cows to ensure that the animals used are in fact a truly representative sample. For such experiments, therefore, it has generally been found preferable to select suitable pairs† of unrelated cows, similar as far as possible in breed, age, stage of lactation, stage of gestation, physical condition, milk yield and milk composition, and where possible to check on the extent of these similarities by a control run before beginning the experimental treatment. It has been found preferable also so to plan the experiment that differences between the cows in the selected pairs can be allowed for in analysing the results. When the monozygous twins are to be employed for investigations of this type, computation of 'twin efficiency values' for a limited number of sets of twins selected completely at random is somewhat artificial—it presents an unduly favourable picture of the value of monozygotic twins for experimental purposes. In this connexion both Hancock and Patchell (*loc. cit.*) have pointed out that estimations of 'twin efficiency values' are subject to large sampling errors and serve mainly to indicate which characteristics may profitably be studied by use of monozygotic twins.

Most of the computations of 'twin efficiency values' have been made on whole-lactation averages, and give results which are of interest primarily to the geneticist. For the

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† As is well known, it is difficult to pair unrelated cows satisfactorily for more than two or three characteristics, whereas monozygous twins are already paired for a large number of characteristics.

manufacturer of milk products, however, the main interest is in short-term fluctuations in milk and butterfat composition and in the causes of these fluctuations. The main object in a uniformity trial with monozygotic twins to be used in investigations on these aspects of milk production is therefore to study the uniformity of the reaction of the cows in each twin set to changes in feed, environment and stage of lactation compared with that of the reaction of unrelated cows of a type which, in default of a supply of monozygous twins, might be used for the experimental work. Such a trial would involve (*a*) comparison of uniformity of the results for a selected number of monozygous twins with results from a similar number of paired animals, and (*b*) presentation of the individual analytical results throughout the complete lactation rather than whole-lactation averages. In respect to factor (*a*) the present report suffers from the same deficiency as those of Hancock and of Patchell in that within-twin-set results are compared only with between-twin-set results—it was not possible to extend the trial to comparisons of results for selected paired cows kept under the same conditions. In respect to factor (*b*), Hancock (3) has given results for daily yields of milk, butterfat and casein, and for daily percentages of butterfat and casein* in the milk, and of daily casein/fat ratios, for a number of monozygous twins at 28-day intervals over a lactation period. He found that within some sets of twins there was a uniform relationship between the results over the whole lactation period, but within some other sets the twin pair mates did not have the same ranking order on all sampling days. Computation of the results on a whole-lactation basis would thus introduce compensation of within-twin-set variations.

The main purpose of the present paper is to report the results of analysis of butterfats from some of the cows used by Patchell (4) in the uniformity trial already referred to. Since it is important to study the within-twin uniformity over as wide a range of data as possible, and since Patchell did not present in detail the basic data on which his study was made, results for body weight, milk yield, fat percentage and fat yield, solids-not-fat content and solids-not-fat yield, lactose content and protein content for the cows used in the butterfat investigation have been included.

*Changes in butterfat characteristics.* The iodine value, saponification value, Reichert value and softening point (Cox & McDowall (5)), the nature of the unsaturated fatty acids (McDowell (6)), the density and refractive index (McDowell (7)), and the carotene and vitamin A contents (McDowell & McDowall (8)) of New Zealand butterfat have been shown to vary considerably over a season. The changes are similar in pattern for butterfats from all North Island districts, and they occur consistently at approximately the same time each year. Because in New Zealand dairying for butter manufacture is mainly seasonal, with most of the lactations commencing in July–August, i.e. in late winter or early spring, and ending in May–June, i.e. in the subsequent early winter, the changes in butterfat properties are concurrent each year with (*a*) progress of the period of lactation of most of the lactating cows, and (*b*) the normal sequence of changes in open-grazing feeding conditions resulting from the effects of both season of the year and variation in climate during the season on the botanical composition of the pasture and on the nature of the pasture growth and fodder crops.

It has not yet been possible to differentiate the effects of (*a*) from those of (*b*), i.e. to determine how much of the recorded changes in butterfat properties is due to lactational and how much to nutritional influences. An investigation on this problem requires the

* The casein was estimated by formol titration only.

staggering of the calving dates of paired cows so that comparison can be made of butterfats from cows at different stages of lactation grazed on the same pasture.* Because of the wide variation in the properties of butterfat from cows of the same breed, but of no particular genetic relationship, a large number of pairs of animals of this type would be required. For such a trial the necessary extensive analytical facilities were not available. It seemed probable that with monozygotic twins the numbers required would be much smaller.

An investigation of the properties of butterfat from twin cows has been reported by Hancock (3), based on analyses made at this Institute. The 'twin efficiency values' computed by Hancock, viz. iodine value 4, saponification value 1, Reichert value 11 and softening point 9, indicated a considerably greater uniformity in the characteristics of butterfat from twin cows than in those of butterfat from unrelated cows kept under the same feeding conditions. Hancock's results, however, refer to only three samples of butterfat from each cow of four pairs of twins, and the samples were taken at weekly intervals over three successive weeks at the middle of the lactation period. The number of twins used, the number of samples analysed, and the range of the period of sampling, were thus small. Because of these limitations the results do not provide a satisfactory indication of the extent of the advantages to be gained, in work on the cause of changes in butterfat characteristics, from using monozygotic twins rather than paired unrelated cows. The present report covers an investigation of the variation in properties of 3-day composite samples of butterfat collected at fortnightly intervals from seven pairs of monozygous twins kept under open-grazing conditions on the Institute farm over a whole lactation during the 1951–52 dairying season.

## EXPERIMENTAL MATERIAL AND METHODS

*Breed and calving dates of cows.* The cows (see Table 1) were seven of the ten sets of twins used by Patchell in his uniformity trial on composition of milk from twin cows (4). They were all of Jersey or Jersey-cross breed (see Table 1). Four sets were 3-year-old

Table 1. *Description of seven pairs of twin cows used in the investigation*

| Twin cow no. | Breed | Calving date (1951) | Age at calving | | Body weight | |
|---|---|---|---|---|---|---|
| | | | yr. | days | At calving (lb.) | At 252 days after calving (lb.) |
| T15 | Jersey/Red-Poll | 30. vii. | 3 | 344 | 957 | 1080 |
| T16 | | 6. viii. | 3 | 351 | 890 | 1005 |
| T17 | Jersey/Jersey-Polled Angus | 9. viii. | 4 | 312 | 903 | 958 |
| T18 | | 13. viii. | 4 | 316 | 927 | 976 |
| T25 | Jersey/Jersey-Shorthorn | 31. vii. | 3 | 57 | 874 | 1060 |
| T26 | | 22. vii. | 3 | 48 | 819 | 940 |
| T49 | Grade Jersey | 1. viii. | 2 | 329 | 697 | 792 |
| T50 | | 20. vii. | 2 | 317 | 744 | 793 |
| T53 | Grade Jersey | 10. viii. | 4 | 333 | 855 | 973 |
| T54 | | 14. vii. | 4 | 306 | 880 | 986 |
| T71 | Grade Jersey | 1. viii. | 2 | 322 | 697 | 762 |
| T72 | | 5. viii. | 2 | 326 | 625 | 743 |
| T89 | Jersey/Ayrshire | 22. vii. | 2 | 356 | 768 | 904 |
| T90 | | 16. vii. | 2 | 350 | 757 | 913 |

* A trial of this type with monozygous twins is now in progress.

heifers at time of calving, one set was 4-year-old cows, and two sets were 5-year-old cows. The calving dates for the cows in the twin pairs were within 12 days of each other, except for cows T53 and 54, for which they were 27 days apart (see Table 2). (The cows were judged to be monozygous twins on the basis of their morphology and of close observation of the changes that occurred during their development.)

Table 2. *Comparison of ages and body weights of cows within each twin pair*

| Twin cow no. | Difference* between ages at calving (days) | Difference* (lb.) between body weights | | Increases (lb.) in body weights | |
|---|---|---|---|---|---|
| | | At calving | At 252 days after calving | Over 252 days | Difference* |
| T15 | 7 | −67 | −75 | 123 | −8 |
| T16 | | | | 115 | |
| T17 | 3 | +24 | +18 | 55 | −6 |
| T18 | | | | 49 | |
| T25 | 9 | −55 | −120 | 186 | −65 |
| T26 | | | | 121 | |
| T49 | 12 | +47 | +1 | 95 | −46 |
| T50 | | | | 49 | |
| T53 | 27 | +25 | +13 | 118 | −12 |
| T54 | | | | 106 | |
| T71 | 4 | −72 | −19 | 65 | +53 |
| T72 | | | | 118 | |
| T89 | 6 | −11 | +9 | 136 | +20 |
| T90 | | | | 156 | |

* The plus and minus signs indicate whether the result for the even-numbered twin cow was greater (+) or less (−) than that for her odd-numbered twin mate.

*Body weights.* There was a considerable difference in the weights of the cows in a number of the twin pairs, both at calving and at completion of lactation (see Table 2). This difference, where it was appreciable, persisted in the same direction throughout the lactation (see Fig. 1). All the cows showed the usual considerable increase in weight during lactation (Flux & Patchell (9)), but for the individual cows of the twin pairs there were differences in the extent of this increase (see last two columns of Table 2, and Fig. 1). Further reference to these changes in body weight will be made later during the discussion of the analytical results.

*Feeding.* The cows were fed on pasture throughout the experimental period. During periods of pasture shortage, particularly over the summer months, they were given, as supplements, some grass silage and choumoellier (marrow-stem kale).

*Sampling of butterfats.* The butterfats were prepared from milks collected at six successive milkings over 72 hr. each fortnight throughout the period of sampling. The dates of sampling corresponding to the sampling period code numbers used in the graphs are given in Table 3. Forty ounces of milk from each evening milking were held overnight and mixed with 40 oz. from the subsequent morning milking.* The combined sample was passed through a cream separator after the morning milking. The creams were collected

* Because of the differences between characteristics of butterfats from the night and morning milkings (Mayhead & Barnicoat (10)), this system of sampling, in which differences in yields of milk at the evening and morning milkings were ignored, would introduce a small error. Since, however, the diurnal variations in properties of butterfat are fairly uniform, the error would apply for all cows and would not appreciably affect the comparison.

Table 3. *Dates corresponding to sampling period code numbers (see Figs. 1–16)*

(Date is given for last morning of six-milking sampling period.)

| Code no. | Date | Code no. | Date | Code no. | Date |
|---|---|---|---|---|---|
| 1 | 7–9. viii. 51 | 8 | 13–15. xi. 51 | 15 | 19–21. i. 52 |
| 2 | 21–23 viii. 51 | 9 | 27–29. xi. 51 | 16 | 5–7. iii. 52 |
| 3 | 4–6. ix. 51 | 10 | 11–13. xii. 51 | 17 | 18–20. iii. 52 |
| 4 | 18–20. ix. 51 | 11 | 25–27. xii. 51 | 18 | 1–3. iv. 52 |
| 5 | 2–4. x. 51 | 12 | 8–10. i. 52 | 19 | 15–17. iv. 52 |
| 6 | 16–18. x. 51 | 13 | 22–24. i. 52 | 20 | 28. iv.–1. v. 52 |
| 7 | 29. x.–1. xi. 51 | 14 | 5–7. ii. 52 | 21 | 13–15. v. 52 |

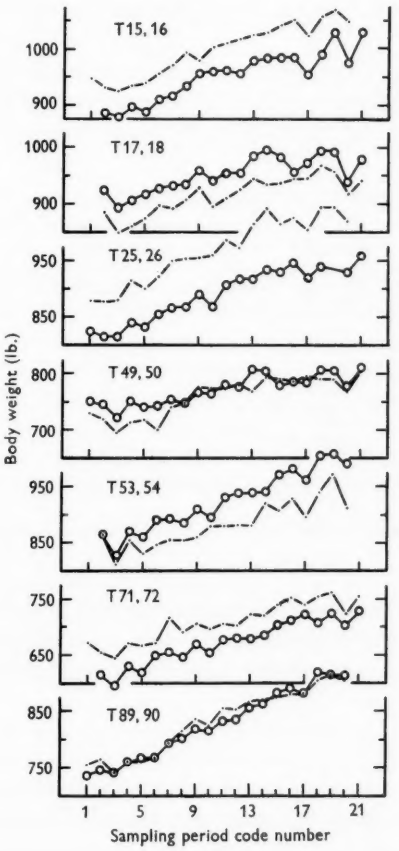


Fig. 1

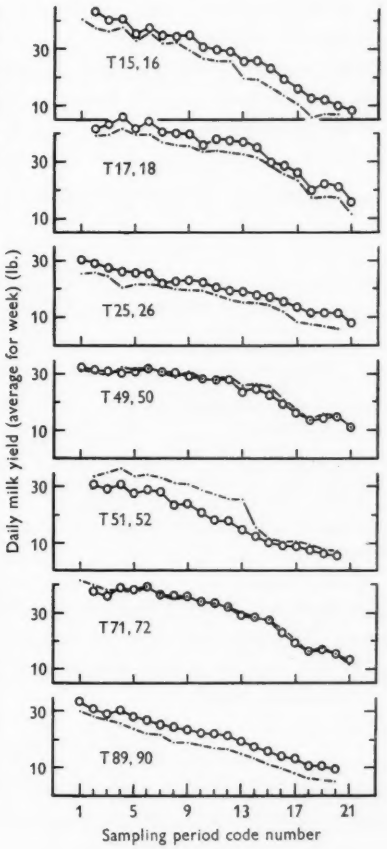


Fig. 2

Fig. 1. Body weights of the twin cows at fortnightly intervals throughout the lactation period. For the meaning of the sampling period code numbers, see Table 3. –·–·, odd-numbered cow; – o – o, even-numbered cow.

Fig. 2. Daily milk yields (weekly averages) of the twin cows at fortnightly intervals throughout the lactation period. –·–·, odd-numbered cow; – o – o, even-numbered cow.

into one container for each cow at the successive separations over the 3-day period. During the sampling period, and thereafter until required for churning, the creams were held in a refrigerator at 40° F. Over the whole lactation period the maximum number of samples taken from any one cow was 21. Results for all these samples are shown in the graphs, but in the tables results for only nineteen sampling periods (periods 2–20) are given, since only results from sampling dates applicable for all the cows could be utilized for computation of the means.

*Preparation of butterfat sample and methods of analysis.* The creams were churned in a laboratory churn (Dolby (11)) and butterfat was isolated from the butter by melting and filtering. Iodine values, saponification values and Reichert values were estimated by the British Standard methods (12). Softening points were determined by Barnicoat's modification of the falling-ball method (13). Refractive index was measured by means of an Abbé refractometer at 40° C. Carotene and vitamin A were estimated by methods already described (McDowell (14)).

*Analytical errors.* Since the main interest of the results is in the magnitude of the differences for the cows in each twin pair—differences which can be expected not to be large—it is important to know the range of error of results for the methods of analysis

Table 4. *Errors of estimation of results for butterfat properties*

| Butterfat property | Average divergence between duplicates | Maximum divergence between duplicates | Standard error of single estimation | Standard error of means of duplicate estimations* |
|---|---|---|---|---|
| Iodine value | 0·25 | 0·8 | 0·14 | 0·10 |
| Saponification value | 0·60 | 2·0 | 0·39 | 0·28 |
| Reichert value | 0·17 | 0·5 | 0·10 | 0·07 |
| Softening point (° C) | 0·09 | 0·40 | 0·06 | 0·45 |
| Refractive index $n_D^{40°}$† | — | — | — | — |
| Carotene (μg./g.) | 0·24 | 0·8 | 0·16 | 0·11 |
| Vitamin A (μg./g.) | 0·09 | 0·7 | 0·13 | 0·09 |

* Two-thirds of the estimations of means of duplicates would be expected to fall within the range of the mean plus this figure to the mean minus this figure.

† The refractive index values recorded were the averages of eight readings and can be accepted as accurate to within ±0·0001.

used. The figures in Table 4 have been computed from the duplicate results of the estimations on the properties of the fat over the whole experimental period. In studying the graphs (Figs. 5–16) the possible range of error of the means of the duplicate estimations should be borne in mind.

## RESULTS

*Annual milk and butterfat production.* The annual milk and butterfat production and the weighted mean annual fat percentages of the milks are given in Table 5. The within-twin-set variations were proportionately greater for milk yield than for the butterfat percentages. Except for the two twin sets, T49, 50 and T71, 72, for which the differences in annual milk yields were small,the cow of each twin set with the lower annual average milk yield gave the milk that had the higher annual average fat percentage. The within-twin-set difference in butterfat yield was thus smaller in proportion than the within-twin-set difference in milk yield.

Table 5. *Yields of milk and butterfat, and butterfat percentages (see also Figs. 2, 3 and 5)*

(Results calculated on an annual basis, 252-day lactation period.)

| Twin cow no. | Milk yield | | Butterfat yield | | Butterfat percentage | |
|---|---|---|---|---|---|---|
| | Total (lb.) | Difference* (lb.) | Total (lb.) | Difference* (lb.) | Annual average (%) | Difference* (%) |
| T15 | 6808 | +779 | 340 | +14 | 4·99 | −0·32 |
| T16 | 7587 | | 354 | | 4·67 | |
| T17 | 8218 | +902 | 257 | +28 | 3·13 | −0·01 |
| T18 | 9120 | | 285 | | 3·12 | |
| T25 | 4463 | +1210 | 258 | +47 | 5·54 | −0·16 |
| T26 | 5673 | | 305 | | 5·38 | |
| T49 | 6897 | +53 | 389 | +4 | 5·64 | +0·02 |
| T50 | 6950 | | 393 | | 5·66 | |
| T53 | 6301 | −698 | 274 | −2 | 4·35 | +0·51 |
| T54 | 5603 | | 272 | | 4·86 | |
| T71 | 8276 | −301 | 386 | −14 | 4·66 | 0·00 |
| T72 | 7975 | | 372 | | 4·66 | |
| T89 | 4911 | +1183 | 213 | +43 | 4·33 | −0·12 |
| T90 | 6094 | | 256 | | 4·21 | |

* The algebraical signs indicate the direction of the divergence of the result for the even-numbered twin cow from the result for her odd-numbered twin mate.

*Daily milk yield.* The average daily milk yields for each cow during the weeks when the 3-day milk samples were taken each fortnight are given in Fig. 2. All the graphs have the form of the usual lactational change in milk yield.

*Relationship between differences in body weight and differences in milk yield.* The cows of the two twin pairs, T49, 50, and T89, 90, had very uniform within-twin-set body weights throughout the lactation (Fig. 1). Cows T49, 50 had also uniform milk yields (Fig. 2), whereas for cows T89, 90 there was a difference in milk yield, the yield for T90 being consistently the higher throughout. On the other hand, cows T71, 72 showed a consistent difference in body weight throughout the lactation period, but a very close correspondence in milk yield. For cows T15, 16, T17, 18, T25, 26 and T53, 54 there was a considerable difference in body weight during the whole experimental period, but in some pairs of these twins the heavier cow gave the higher milk yield and in other pairs the lighter cow gave the higher milk yield. There was thus no consistent relationship between difference in body weight and within-twin-set difference in milk yield (see also (4)).

*Fat content of milk and fat yield* (see Figs. 3, 5). For twin pairs T49, 50 and T71, 72 there was a close within-twin-set relationship for both the fat content of the milk and the yield of fat. The consistent difference in body weights for T71, 72 (Fig. 1) did not cause any corresponding differences in these two values. Although the within-twin-set divergences in the graphs for all seven sets of twins were much smaller than the between-twin-set divergences (see the scatter diagrams at the bottom of Figs. 3 and 4), the within-twin-set divergences for some sets of twins were appreciably and consistently greater than for other sets (compare T53, 54 with T71, 72—Figs. 3, 5).

*Solids-not-fat content of milk and solids-not-fat yield* (see Figs. 4 and 6). The comments in the preceding paragraph on variation in fat content and fat yield apply also to the

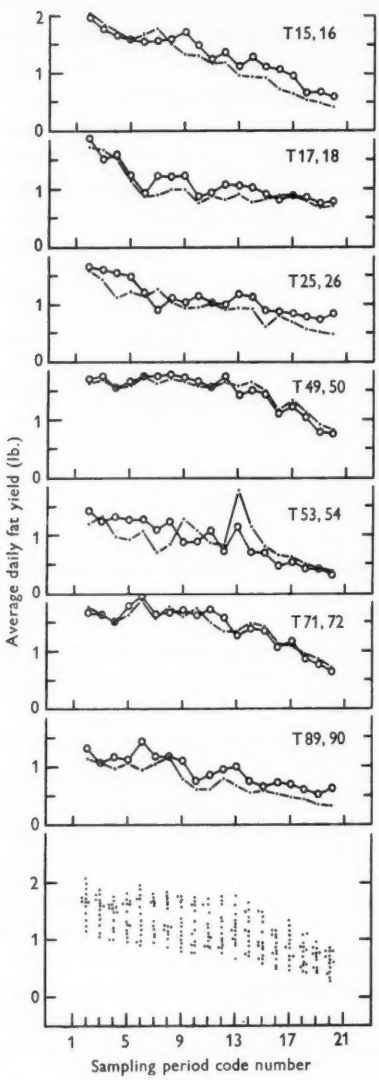


Fig. 3

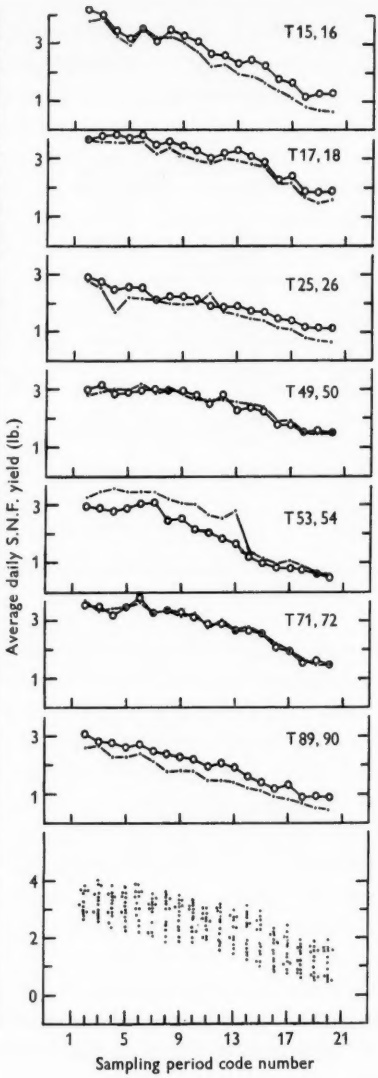


Fig. 4

Fig. 3. Mean daily fat yields of the twin cows over 3-day periods at fortnightly intervals throughout the lactation period. –·–·, odd-numbered cow; – o – o, even-numbered cow.

Fig. 4. Mean daily solids-not-fat yields of the twin cows over 3-day periods at fortnightly intervals throughout the lactation period. –·–·, odd-numbered cow; – o – o, even-numbered cow.

solids-not-fat content of the milk and to the solids-not-fat yield. The consistent difference in body weight for cows T71 and 72, for example, was not associated with any consistent difference in solids-not-fat content of the milk or in solids-not-fat yield, and the close similarity in body weights for cows T89, 90 did not prevent very considerable differences in solids-not-fat contents of the milks.

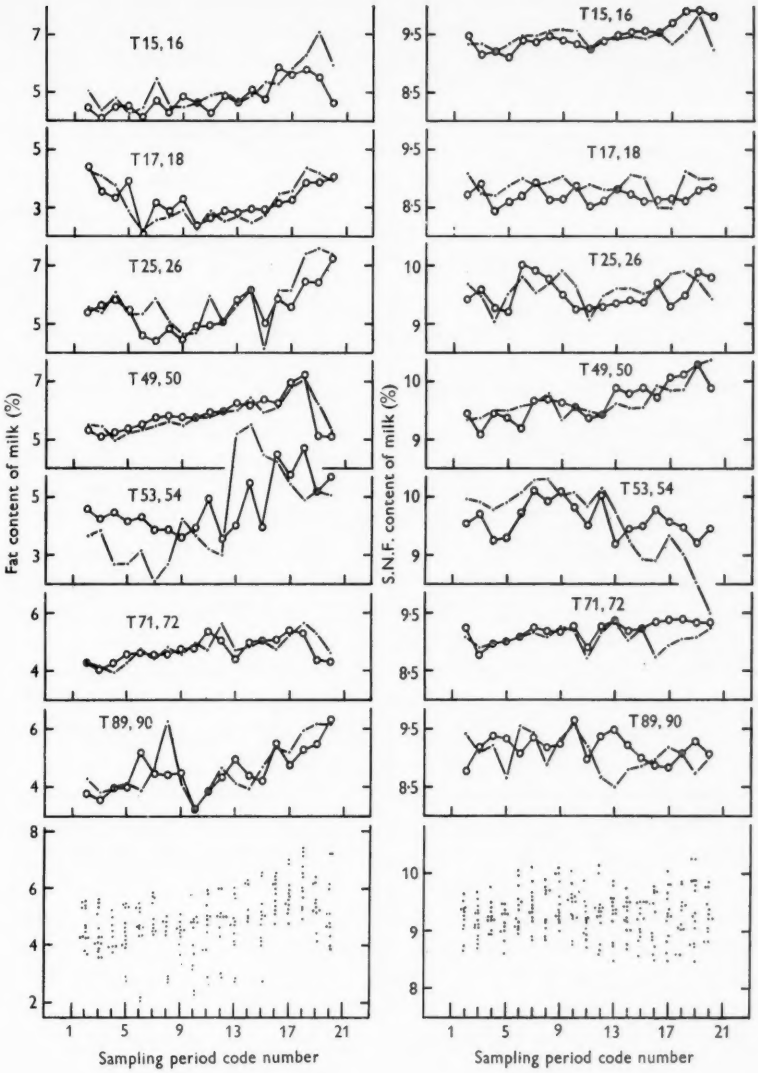


Fig. 5 Fig. 6

Fig. 5. Fat contents of 3-day composite samples of milks from the twin cows at fortnightly intervals throughout the lactation period. – · – ·, odd-numbered cow; – o – o, even-numbered cow.

Fig. 6. Solids-not-fat contents of 3-day composite samples of milks from the twin cows at fortnightly intervals throughout the lactation period. – · – ·, odd-numbered cow; – o – o, even-numbered cow.

*Lactose content.* The results for lactose content are given in Fig. 7. In their general form the lines are typical lactation curves for the lactose content of milk, i.e. fairly uniform results over the first few months and a fall towards the end of lactation. The trends of the curves have a greater similarity within-twin-sets than between-twin-sets. The close agreement over the whole lactation period for cows T49, 50 was particularly

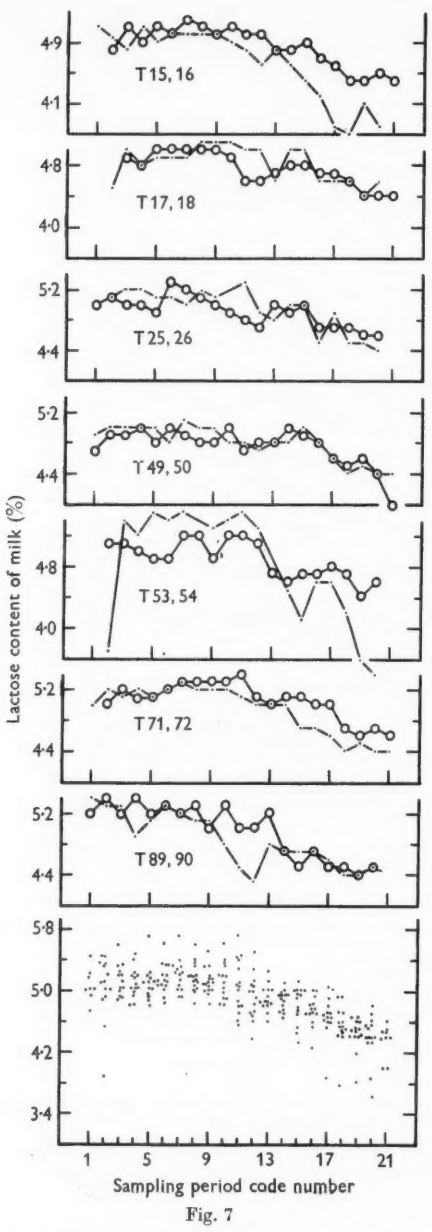


Fig. 7

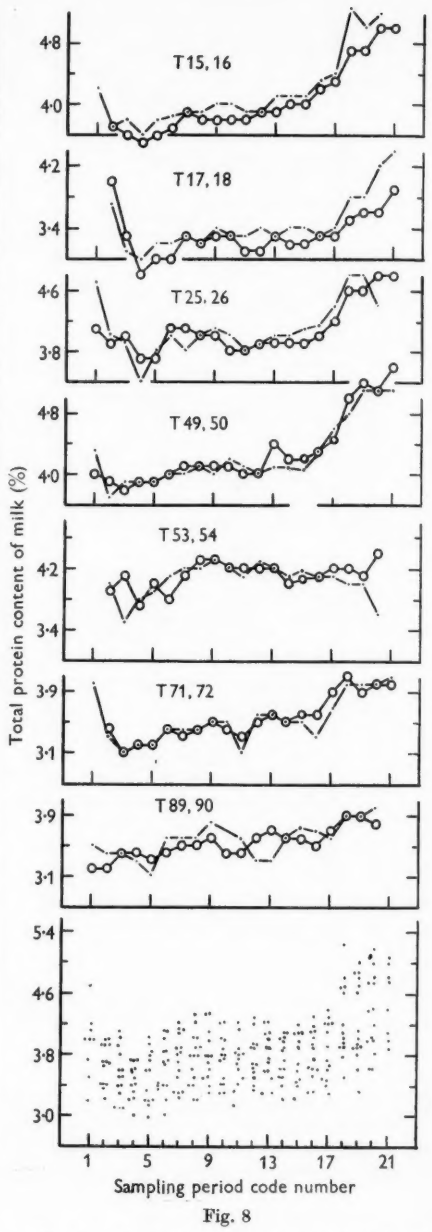


Fig. 8

Fig. 7. Lactose contents of 3-day composite samples of milks from the twin cows at fortnightly intervals throughout the lactation period. – · – ·, odd-numbered cow; – o – o, even-numbered cow.

Fig. 8. Total protein contents (N × 6·38) of 3-day composite samples of milks from the twin cows at fortnightly intervals throughout the lactation period. – · – ·, odd-numbered cow; – o – o, even-numbered cow.

noticeable. There were, however, some wide within-twin-set divergences, e.g. the rapid falling away in lactose content at the end of the lactation period for cows T15 and T53 compared with the corresponding values for their twin sisters. (The difference was not due to difference in calving dates, see Table 1.) It is evident that use of monozygotic twins rather than unrelated cows is not likely to bring any great advantage in short-term investigations of causes of change in lactose content of milk (compare the low value, 1·2, computed by Patchell (4) for the 'twin efficiency value' based on the average annual lactose content).

Comparison of Fig. 7 with Figs. 1 and 2 does not reveal any relationship between the within-twin-set variation in lactose content and the within-twin-set variation in body weight or milk yield for the same sampling period.

*Total protein content.* The results are given in Fig. 8. The general trend of the changes was characteristic of the normal lactation curve for total protein content of milk, i.e. a fall early in lactation and a rise towards the end. For the cows of each twin set the curves were similar in form throughout, but among the seven twin sets there was variation in the detailed form of the curve. The consistent rise in protein content with progress of lactation for cows T15, 16 and for cows T49, 50 over sampling periods 15–21 was in sharp contrast with the very small change for cows T53, 54; and the close agreement between the results for the cows of each twin set is in sharp contrast with the spread of the results for all the cows, as shown in the scatter diagram at the bottom of Fig. 8.

The 'twin efficiency value' computed by Patchell (4) for total protein content, based on whole-lactation averages for ten sets of twins (including the seven sets used in the present investigation), was 12·6.

Comparison of Figs. 1 and 2 with Fig. 8 does not reveal any relationship between within-twin-set differences in body weight or in yield of milk and differences in the protein content of the milk. For cows T71, 72, for example, there was a difference in body weight during the whole lactation but the protein contents of the milks were similar throughout the period.

*Butterfat characteristics.* The unweighted* means for nineteen sampling periods (nos. 2–20, Table 3) for each of the fourteen cows are given in Table 6. The ranges of variation are shown at the end of the table.

*Between-twin-set variations—annual unweighted means.* These are given in Table 7. Comparison of the range of variation for all the results (given at the end of Table 6) with the range of variation for the averaged results in each twin set (given at the end of Table 7) shows that averaging the results for the members of each twin set did not alter the range of variation appreciably. The major portion of the range of divergence between the average annual unweighted mean results was thus a between-twin-set variation.

*Comparison of within-twin-set and between-twin-set differences.* (*a*) *Annual unweighted mean results.* In Table 8 the within-twin-set differences for the annual unweighted means are given, and at the end of the table the maximum ranges of variation within twins is compared with the total ranges of variation for the fourteen cows. It is evident that the within-twin-set differences of the unweighted means were much smaller than the between-twin-set differences.

(*b*) *Single sampling period analysis results.* These are shown in Figs. 9–16, separately for each of the seven twin sets, and in the lower part of the figure they are shown for all fourteen cows as a scatter diagram. For all the characteristics studied it is evident that

* For weighted means see Table 9.

Table 6. *Properties of butterfats from the experimental cows*

(Figures represent means of results of estimations on nineteen samples of butterfat collected over 3 days at fortnightly intervals over the lactation period.)

| Twin cow no. | Iodine value | Saponification value | Reichert value | Softening point (° C.) | $n_D^{40°}$ | Carotene content (μg./g.) | Vitamin A content (μg./g.) | Vitamin A potency (i.u./g.) |
|---|---|---|---|---|---|---|---|---|
| T15 | 38·4 | 231·7 | 30·1 | 30·6 | 1·4551 | 8·1 | 8·3 | 41·2 |
| T16 | 37·6 | 232·8 | 29·8 | 30·5 | 1·4550 | 8·4 | 8·1 | 40·6 |
| T17 | 38·0 | 230·9 | 29·0 | 31·6 | 1·4551 | 10·8 | 8·2 | 46·1 |
| T18 | 36·8 | 231·7 | 29·3 | 31·9 | 1·4549 | 11·3 | 8·2 | 47·1 |
| T25 | 35·6 | 230·5 | 27·1 | 32·9 | 1·4548 | 10·6 | 7·6 | 42·4 |
| T26 | 34·7 | 231·7 | 26·7 | 32·8 | 1·4546 | 9·7 | 7·4 | 39·9 |
| T49 | 33·5 | 231·2 | 28·1 | 33·4 | 1·4545 | 6·8 | 7·9 | 37·0 |
| T50 | 33·0 | 231·9 | 28·0 | 33·4 | 1·4545 | 7·1 | 7·6 | 36·5 |
| T53 | 34·2 | 231·8 | 28·0 | 33·2 | 1·4546 | 7·7 | 8·0 | 39·3 |
| T54 | 35·8 | 230·3 | 26·7 | 33·5 | 1·4549 | 8·6 | 8·6 | 42·8 |
| T71 | 35·2 | 231·4 | 28·3 | 32·4 | 1·4547 | 9·6 | 7·8 | 42·3 |
| T72 | 35·5 | 232·0 | 28·7 | 32·4 | 1·4547 | 10·0 | 7·5 | 41·8 |
| T89 | 37·9 | 228·6 | 28·1 | 33·5 | 1·4552 | 7·6 | 7·7 | 38·5 |
| T90 | 37·5 | 229·2 | 28·5 | 33·3 | 1·4552 | 8·2 | 7·5 | 38·7 |
| Minimum | 33·0 | 228·6 | 26·7 | 30·5 | 1·4545 | 6·8 | 7·5 | 36·5 |
| Maximum | 38·4 | 232·8 | 30·1 | 33·5 | 1·4552 | 11·3 | 8·6 | 47·1 |

Table 7. *Properties of butterfats from the twin pairs*

(Figures represent averages of results, given in Table 6, for each twin pair)

| Twin cow no. | Iodine value | Saponification value | Reichert value | Softening point (° C.) | $n_D^{40°}$ | Carotene content (μg./g.) | Vitamin A content (μg./g.) | Vitamin A potency (i.u./g.) |
|---|---|---|---|---|---|---|---|---|
| T15, 16 | 38·0 | 232·3 | 30·0 | 30·6 | 1·4551 | 8·3 | 8·2 | 40·9 |
| T17, 18 | 37·4 | 231·3 | 29·2 | 31·8 | 1·4550 | 11·1 | 8·2 | 46·6 |
| T25, 26 | 35·2 | 230·9 | 26·9 | 32·9 | 1·4547 | 10·2 | 7·5 | 41·2 |
| T49, 50 | 33·3 | 231·6 | 28·1 | 33·4 | 1·4545 | 7·0 | 7·8 | 36·8 |
| T53, 54 | 35·0 | 231·1 | 27·4 | 33·4 | 1·4548 | 8·7 | 8·3 | 41·1 |
| T71, 72 | 35·4 | 231·7 | 28·5 | 32·4 | 1·4547 | 9·8 | 7·7 | 42·0 |
| T89, 90 | 37·7 | 228·9 | 28·3 | 33·4 | 1·4552 | 7·9 | 7·6 | 38·6 |
| Minimum | 33·3 | 228·9 | 26·9 | 30·6 | 1·4545 | 7·0 | 7·6 | 36·8 |
| Maximum | 38·0 | 232·3 | 30·0 | 33·4 | 1·4552 | 11·1 | 8·3 | 46·6 |

Table 8. *Comparison of properties of butterfats from individual cows in twin pairs*

(Figures represent differences between the averages cited in Table 6 of fortnightly values for year.)

| Twin cow no. | Iodine value | Saponification value | Reichert value | Softening point (° C.) | $n_D^{40°}$ | Carotene content (μg./g.) | Vitamin A content (μg./g.) | Vitamin A potency (i.u./g.) |
|---|---|---|---|---|---|---|---|---|
| T15, 16 | 0·8 | 1·1 | 0·3 | 0·1 | 0·0001 | 0·3 | 0·2 | 0·6 |
| T17, 18 | 1·2 | 0·8 | 0·3 | 0·3 | 0·0002 | 0·5 | 0·0 | 1·0 |
| T25, 26 | 0·9 | 1·2 | 0·4 | 0·1 | 0·0002 | 0·9 | 0·2 | 2·5 |
| T49, 50 | 0·5 | 0·7 | 0·1 | 0·0 | 0·0000 | 0·3 | 0·3 | 0·5 |
| T53, 54 | 1·6 | 1·5 | 1·3 | 0·3 | 0·0003 | 0·9 | 0·6 | 3·5 |
| T71, 72 | 0·3 | 0·6 | 0·4 | 0·0 | 0·0000 | 0·4 | 0·3 | 0·5 |
| T89, 90 | 0·4 | 0·6 | 0·4 | 0·2 | 0·0000 | 0·6 | 0·2 | 0·2 |
| Minimum | 0·3 | 0·6 | 0·1 | 0·0 | 0·0000 | 0·3 | 0·0 | 0·2 |
| Average | 0·8 | 0·9 | 0·5 | 0·15 | 0·0001 | 0·6 | 0·3 | 1·3 |
| Maximum | 1·6 | 1·5 | 1·3 | 0·3 | 0·0003 | 0·9 | 0·6 | 3·5 |
| Total range for 14 cows | 5·4 | 4·2 | 3·4 | 3·0 | 0·0007 | 4·5 | 1·1 | 10·6 |

the within-twin-set variations were much smaller than the between-twin-set variations, consistently throughout the sampling period.

*Iodine values* (Fig. 9). The seasonal trend evident in the scatter diagram corresponds with that reported by Cox & McDowall (5) for North Island butterfats, but this trend was not shown equally distinctly for all the cows. For cows T25, 26, for example, there was not the distinct fall in iodine value expected for late-spring/early-summer (periods no. 5–7) North Island butterfat; and for cows T25, 26, T53, 54 and T89, 90 the usual autumn rise in iodine value was shown much more distinctly than for the other four pairs. As against these differences in the character of the curves for the different twin sets the results for the two cows of the twin sets showed a remarkable concordance. For the most part the two twin cows reacted in exactly the same way to the change in stage of lactation or in environmental conditions to which they were subjected. They both showed the same peculiarity of change with season, even when this change diverged from the normal season trend. For twin sets T49, 50, T71, 72 and T89, 90, the iodine values were very close throughout. For T53, 54 the result for the odd-numbered cow was consistently lower than that for the even-numbered cow, but the difference remained fairly uniform during the whole sampling period, and the two cows reacted in a remarkably similar way to change in external conditions, although the nature of the reaction was widely different from that of other sets of twins.

*Refractive index* (Fig. 10). The comments made above on the results for iodine value apply to those for refractive index. The lines for the cows of each twin set followed each other much more closely than those for the different sets of twins. It is known that as a general rule high iodine value in commercial butterfat is correlated with high refractive index (15). This is confirmed for individual cow butterfats by the results shown in Figs. 9 and 10, especially by those for cows T53 and 54. Butterfat from cow T54 had consistently a lower refractive index corresponding to the lower iodine value. But the two sets of graphs show clearly also that the relationship between iodine value and refractive index was not always so close. For cows T71 and 72, for example, the late spring change in iodine value was relatively greater than the corresponding late spring change in refractive index.

*Saponification value and Reichert value* (Figs. 11, 12). The correspondence between the values for the butterfats from the cows of each twin set was close throughout, despite considerable difference in the shapes of the curves for the separate twin sets. It is of interest that the divergences between the iodine values and between the refractive indices for the butterfats of T53 and 54 (Figs. 11, 12) were not accompanied by an appreciable divergence in the saponification and Reichert values.

*Softening point* (Fig. 15). The softening points of the butterfats from the two cows of each twin set were in close agreement. In general, the same trends in the curves were shown at any one sampling period. It is somewhat surprising that the divergence in iodine values for butterfats from T53, 54 (Fig. 11) was not reflected in any appreciable difference in softening point (see also (16)). The rising trend in softening point with progress of the season for cows T53, 54 was in strong contrast with the fairly uniform value throughout the season for cows T25, 26.

*Carotene* (Fig. 13). The correspondence between the carotene contents of the butterfats for the cows of each twin set was less spectacular than for the other characteristics discussed above. In relation to the total range of variation shown in the scatter diagram, however, it was still much closer than for the butterfats from the different sets of twins.

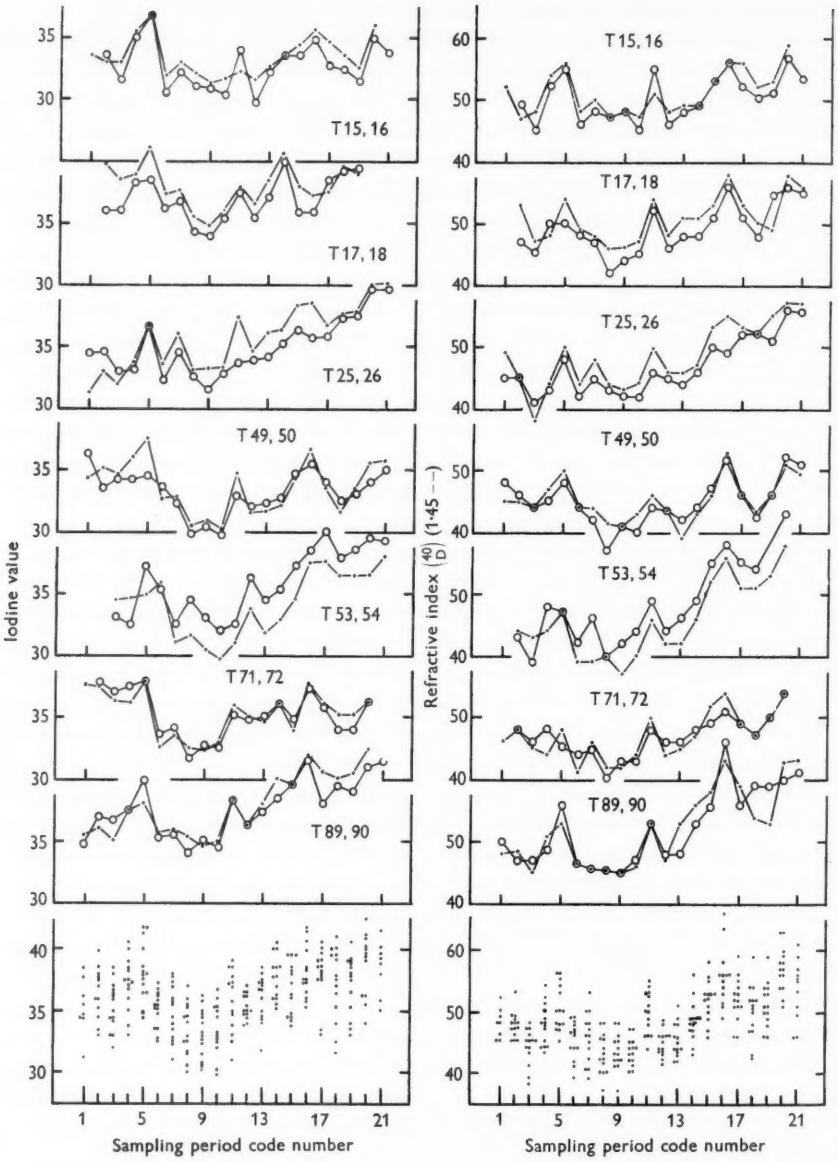


Fig. 9 Fig. 10

Fig. 9. Iodine values of 3-day composite samples of butterfats from the twin cows at fortnightly intervals throughout the lactation period. – · – ·, odd-numbered cow; – o – o, even-numbered cow.

Fig. 10. Refractive indices ($n_D^{40°}$) of 3-day composite samples of butterfat from the twin cows at fortnightly intervals throughout the lactation period. – · – ·, odd-numbered cow; – o – o, even-numbered cow.

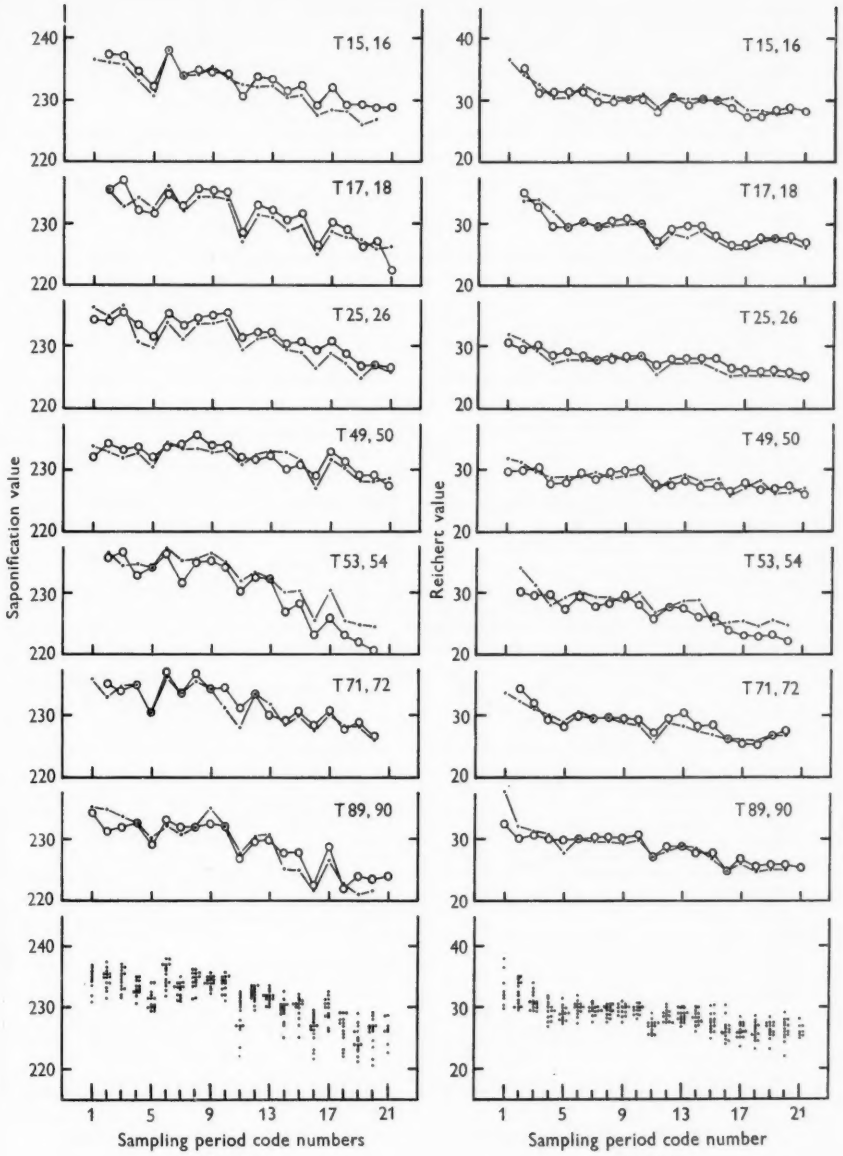


Fig. 11

Fig. 12

Fig. 11. Saponification values of 3-day composite samples of butterfat from the twin cows at fortnightly intervals throughout the lactation period. – · – ·, odd-numbered cow; – o – o, even-numbered cow.

Fig. 12. Reichert values of 3-day composite samples of butterfat from the twin cows at fortnightly intervals throughout the lactation period. – · – ·, odd-numbered cow; – o – o, even-numbered cow.

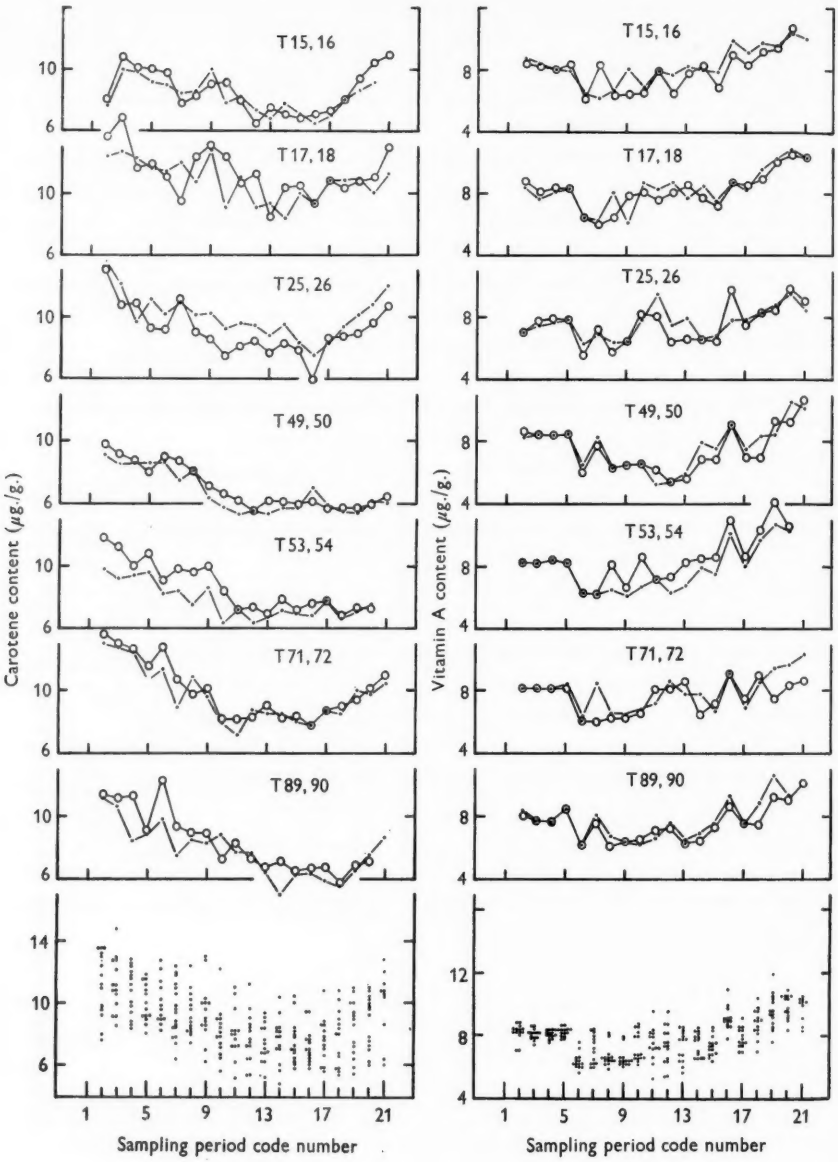


Fig. 13

Fig. 14

**Fig. 13.** Carotene contents of 3-day composite samples of butterfat from the twin cows at fortnightly intervals throughout the lactation period. – · – ·, odd-numbered cow; – o – o, even-numbered cow.

**Fig. 14.** Vitamin A contents of 3-day composite samples of butterfat from the twin cows at fortnightly intervals throughout the lactation period. – · – ·, odd-numbered cow; – o – o, even-numbered cow.

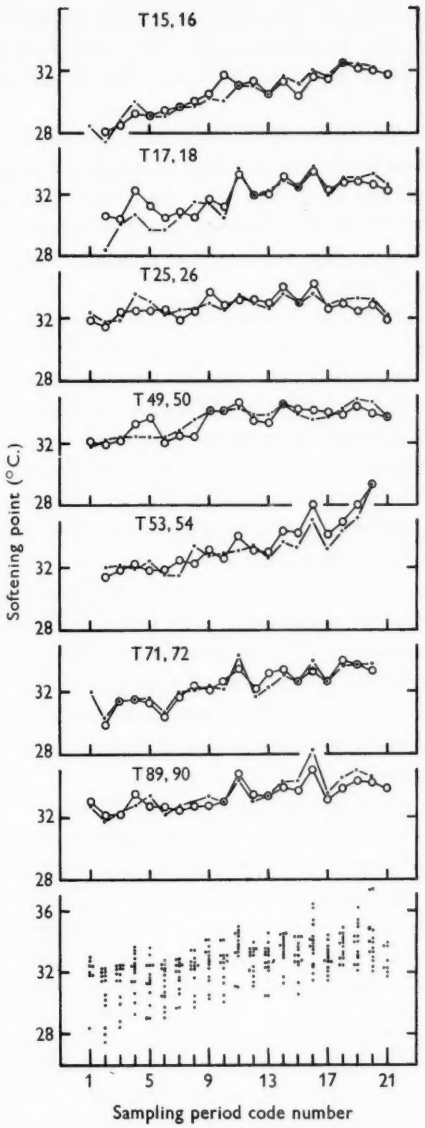


Fig. 15

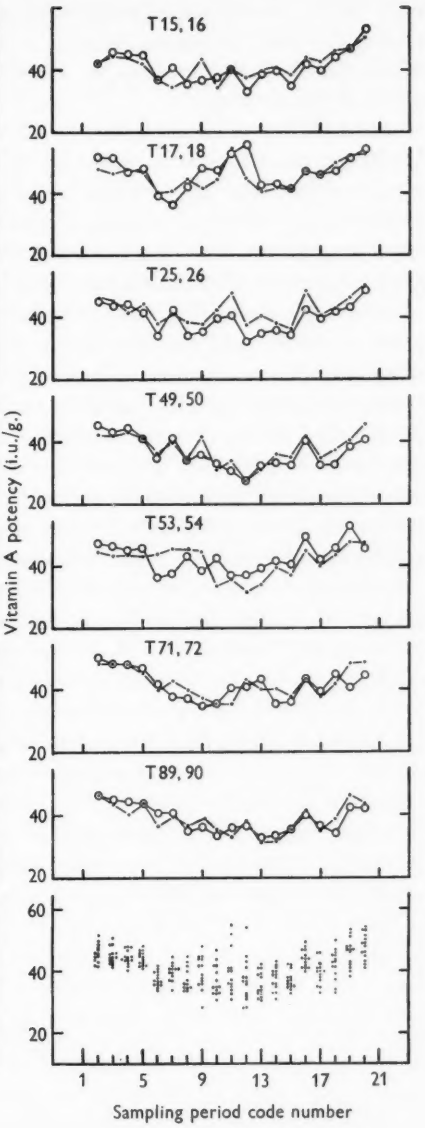


Fig. 16

Fig. 15. Softening points of 3-day composite samples of butterfat from the twin cows at fortnightly intervals throughout the lactation period. – · – ·, odd-numbered cow; – o – o, even-numbered cow.

Fig. 16. Vitamin A potencies of 3-day composite samples of butterfat from the twin cows at fortnightly intervals throughout the lactation period. – · – ·, odd-numbered cow; – o – o, even-numbered cow.

The general seasonal trend was similar to that found by McDowell & McDowall (8) for North Island commercial butterfats, but in some of the other properties referred to above there were differences between twin sets in this respect. For cows T49, 50, T53, 54, and T89, 90, for example, there was no appreciable autumn rise in carotene content of the butterfat.

*Vitamin A* (Fig. 14). The relative spread of the results for the vitamin A content of the milk fat from all the cows, as shown by the scatter diagram in Fig. 14, was smaller than for most of the other characteristics studied; but it is clear from the graphs that the similarity of the vitamin A content of the butterfats from the members of a twin set was greater than for the butterfats from unrelated cows. The trends of the seasonal change in vitamin A content were similar for all the twin sets.

*Vitamin A potency* (Fig. 16). Potency results are derived from the carotene and vitamin A results, and therefore can be expected to reflect the changes noted in these results; but with combination of the two sets of figures there could be some compensation or some accentuation of the differences noted for the individual results. (It is known, for example, that the lower carotene content of Friesian butterfat is counterbalanced by a higher vitamin A content, thus reducing the difference in total vitamin A potency.) The relative spread of the results, as shown by the scatter diagram in Fig. 16, was smaller than for the carotene contents, but the graphs for the twin pairs show clearly that the results were more uniform within-twin-sets than between-twin-sets.

*Consistency of within-twin-set relationships.* As has been pointed out by Hancock (2) monozygous twins are not necessarily identical in every respect. There may be differences originating at the division of the common parent egg-cell, and following conception they may diverge in character to some extent because of differences in environmental influences during the growth and development periods, both pre-natally and post-natally. It is then of interest to inquire how consistent is the consistency or lack of consistency between the cows of the twin sets over a range of characteristics.

From the fortnightly results in Figs. 1–16 it is clear that for cows T49, 50 and T71, 72 the within-twin-set differences were small for all the milk yield and composition data and butterfat properties studied—in other words these twins were very suitable for investigations on all these factors—despite the body-weight differences for cows T71, 72. On the other hand, cows T53, 54 were obviously a poor set of twins for these investigations, and cows T89, 90 although of almost the same body weight throughout, showed very considerable inconsistencies in milk yield and composition and in butterfat properties. With the other three pairs, T15, 16, T17, 18 and T25, 26, there were differences, but these differences remained fairly consistent over the whole period of the investigation.

## STATISTICAL EXAMINATION OF RESULTS FOR BUTTERFAT CHARACTERISTICS*

The weighted lactational averages† for butterfat characteristics, on which the statistical examination was made, are given in Table 9. The analyses of variance for these results, and the between-set and within-set coefficients of variation are given in Table 10. An

* For statistical examination of the milk yield and composition results, see Patchell (4).

† The weighted lactational averages for the butterfat characteristics for each cow, taking iodine value as an example, were calculated from the formula $W = \frac{\Sigma (A \times B)}{\Sigma B}$, where $W$ = the weighted average iodine value, $A$ = the iodine value at a sampling date, and $B$ = the fortnightly yield of fat.

Table 9. *Weighted lactation averages for properties of butterfats from the seven sets of twin cows*

| Twin cow no. | Iodine value | Saponification value | Reichert value | Carotene content (μg./g.) | Vitamin A content (μg./g.) | Vitamin A potency (i.u./g.) |
|---|---|---|---|---|---|---|
| T15 | 38·3 | 233·0 | 30·6 | 9·1 | 8·0 | 40·6 |
| T16 | 37·6 | 233·4 | 30·2 | 9·4 | 8·0 | 41·6 |
| T17 | 38·2 | 231·6 | 29·4 | 11·9 | 8·2 | 44·3 |
| T18 | 36·7 | 231·9 | 29·7 | 12·4 | 8·2 | 46·4 |
| T25 | 35·1 | 231·2 | 27·4 | 10·9 | 7·5 | 42·0 |
| T26 | 34·6 | 232·1 | 27·9 | 10·0 | 7·4 | 40·1 |
| T49 | 33·4 | 231·4 | 28·2 | 7·7 | 7·8 | 37·6 |
| T50 | 33·2 | 232·0 | 27·8 | 7·8 | 7·6 | 37·0 |
| T53 | 33·5 | 233·5 | 28·7 | 8·5 | 7·7 | 38·7 |
| T54 | 35·0 | 232·1 | 27·6 | 9·8 | 8·3 | 42·9 |
| T71 | 35·1 | 231·8 | 28·5 | 10·5 | 7·8 | 42·2 |
| T72 | 35·4 | 232·4 | 29·0 | 11·0 | 7·4 | 41·9 |
| T89 | 37·2 | 230·1 | 28·8 | 8·8 | 7·5 | 38·8 |
| T90 | 37·2 | 229·9 | 28·9 | 9·4 | 7·4 | 39·7 |

Table 10. *Analyses of variance, 'twin efficiency values' and coefficients of variation for weighted mean values of butterfat properties from the seven sets of twin cows*

| Characteristic | Between-set mean square | Within-set mean square | Twin efficiency value | Between-set coefficient of variation | Within-set coefficient of variation |
|---|---|---|---|---|---|
| Iodine value | 6·380 | 0·384 | 7·8 | 3·46 | 1·22 |
| Saponification value | 2·092 | 0·270 | 3·4 | 0·29 | 0·16 |
| Reichert value | 1·833 | 0·153 | 5·5 | 2·26 | 0·97 |
| Carotene | 4·008 | 0·247 | 7·8 | 10·06 | 3·57 |
| Vitamin A | 0·177 | 0·041 | 1·7 | 2·39 | 1·84 |
| Vitamin A potency | 12·880 | 2·120 | 2·5 | 4·00 | 2·50 |

Table 11. *Within-lactation analyses of variance for butterfat composition characteristics*

| | | Mean squares | | | | | |
|---|---|---|---|---|---|---|---|
| Source | Degrees of freedom | Iodine value | Saponification value | Reichert value | Softening point | Carotene content | Vitamin A content |
| Between sets | 6 | 120·03 | 42·97 | 33·33 | 42·41 | 78·52 | 4·39 |
| Within sets | 7 | 8·29 | 9·69 | 3·30 | 0·40 | 3·97 | 1·00 |
| Between periods | 18 | 43·33 | 145·47 | 45·07 | 12·93 | 29·07 | 14·48 |
| Periods × sets | 108 | 2·30 | 2·96 | 1·09 | 0·55 | 1·23 | 0·52 |
| Residual | 113 | 0·65 | 1·15 | 0·49 | 0·26 | 0·57 | 0·28 |
| Total | 265 | — | — | — | — | — | — |

analysis of the results on a within-lactation basis is given in Table 11. Nearly all the mean squares tested in turn against the residual mean squares were highly significant at the 1% level. The exception was the within-set source of variation for softening point, which was non-significant. The respective components of variance, expressed as percentages of the total variance, are given in Table 12.

*Discussion of statistical results.* The between-set differences in iodine value, saponification value, Reichert value and carotene content were highly significant, and the between-set differences in vitamin A and vitamin A potency were significant.

From Table 12 it is clear that the variance due to sampling days contributed a large proportion of the total variance, especially for saponification value, Reichert value and vitamin A content for which the between-set differences accounted for only 6·1, 15·9

Table 12. *Sources of variation in butterfat properties expressed as percentages of the total variance*

| Source | Iodine value | Saponification value | Reichert value | Softening point | Carotene content | Vitamin A content |
|---|---|---|---|---|---|---|
| Between sets | 37·6 | 6·1 | 15·9 | 45·6 | 38·1 | 6·9 |
| Within sets | 5·2 | 3·3 | 3·1 | 0·4 | 3·5 | 0·6 |
| Between periods | 37·9 | 75·3 | 64·7 | 36·8 | 40·6 | 65·8 |
| Periods × sets | 10·6 | 6·7 | 6·2 | 6·3 | 6·5 | 7·8 |
| Residual | 8·7 | 8·6 | 10·1 | 10·9 | 11·3 | 18·9 |
| Twin efficiency value | 7 | 2 | 5 | 109 | 11 | 12 |

and 6·9% of the total variance, respectively. With the seven sets of twins used, therefore, genetical differences were not a major cause of variation in these values. (The finding cannot be extrapolated beyond its application to the seven sets of twins under study to the generalization that these properties are not affected by genetical factors. It is well known, for example, that vitamin A content of butterfat varies markedly with breed of cow. In the group of twin sets investigated there were no Friesian cows.) The highly significant differences due to sampling days was probably the result of the seasonal lactational trends for the properties studied.

The interaction term, periods × sets, was highly significant in all cases, although it reached 10% of the total variance only for iodine value. Since this interaction term was highly significant in the present trial in which all animals were treated alike, it seems possible that under experimental conditions with widely different treatments it could become an important source of variation in the results. Hence the indications gained from uniformity trial 'twin efficiency values' as to numbers of pairs of twins required for an investigation are likely to be too low.

The residual variance, which included the within-set × period variance with the error variance, amounted to 8–11% of the total variance. Errors arising from sampling and analysis thus may have accounted for a fair proportion of the total variance; but since this figure includes within-set × periods interaction, some considerable portion of this residual variance may have been due to the factor referred to in the preceding paragraph. Although it is clear that monozygous twins are greatly superior to unrelated cows for studies on butterfat composition, the magnitude of the residual term indicates that even with these twins replication and frequent sampling throughout the experimental period are necessary in order to keep the error variance as small as possible.

## GENERAL DISCUSSION

The results show that a close approach to identity in body weight is not a reliable index of twin sets that will give a close approach to identity in other characteristics, and difference in body weight is not a reliable index of an unsatisfactory set of twins. While some sets of twins under the same treatment conditions give uniformly parallel results for most characteristics, this does not apply for all sets of twins. A set may show a greater uniformity for some characteristics than for other characteristics. Despite these irregularities, however, the results of the present investigation make it abundantly clear that for work on the causes of change in butterfat characteristics there is a very great advantage in using monozygotic twins rather than unrelated animals, for all the properties studied.

The cows of the twin pair react to changes in stage of lactation and in feeding conditions much more uniformly than do unrelated cows, i.e. the form of the lactation curve is similar for both members of the twin set although it may diverge from the average form of the curve for the property under examination. It should be remembered, however, that there was some heterogeneity in the breed and age of the seven pairs of twins (see Table 1) and that therefore the within-twin/between-twin comparison is somewhat loaded in favour of the within-twin results, and, as stated earlier, the 'twin efficiency values' reported here cannot be accepted as of general applicability. For any experiment with unrelated cows in which the number of cows to be used for the comparison is small, paired cows of similar character would be used. The resemblance among these cows would then probably be closer than the resemblance between the means of the seven sets of twins, and it is probable, although not certain, that the spread of results from such selected cows kept under uniform conditions would be smaller than the means of the results for the seven sets of twins in the present investigation. (This restriction of the experimental population introduced by careful selection of pairs of unrelated cows would impose a limitation on the validity of any generalization reached on the basis of the experimental results.)

The graphs illustrate very clearly that there are differences in the reaction of cows to the factors causing change in butterfat properties with progress of the dairying season; and the demonstration that these differences apply uniformly to both members of a monozygotic twin pair establishes them as almost certainly of genetic origin.

## CONCLUSIONS

It is evident that the usefulness of monozygous twins for studying causes of changes in milk composition and in butterfat characteristics varies with variation in a number of factors:

(*a*) *The characteristic under investigation*—Figs. 1–16 and the twin efficiency values for butterfat characteristics studied show that the similarity of the cows in a twin pair is not equivalent for all these characteristics.

(*b*) *Stage of lactation over which the properties are studied*—the graphs for lactose and protein contents in Figs. 7 and 8 show that twins are most useful for these values during the early months of lactation, and that, particularly for lactose, they are not to be relied upon during the later months of lactation.

(*c*) *Twin-set 'individualities'*—the cows in some sets of twins show a closer approach to the 'identical' than others.

Monozygous twin cows are shown to be much more suitable than unrelated cows for work on causes of change in properties of butterfat, but, because of the above irregularities, it is still desirable to arrange for pre-treatment control periods to provide information on the relationship between the results for the two cows of the twin set under the same conditions of treatment. The similarity of the reaction of the twins to changes in feeding conditions beyond the control of the investigator is then a valuable advantage because of its effect in reducing the difference between the results for the control and experimental cows other than that due to the treatment applied.

SUMMARY

1. Seven sets of monozygous twins were kept under the same open-grazing conditions for one year. Milks were analysed for fat, solids-not-fat, lactose and total protein contents, and butterfats were isolated at fortnightly intervals for estimation of iodine value, refractive index, softening point, saponification value, Reichert value, carotene content and vitamin A content. The results are presented both graphically and statistically.

2. Consistent differences in body weights of the cows of a twin set did not render the twin set unsuitable for use in experimental work on causes of changes in milk yield, fat yield, milk composition or butterfat characteristics.

3. The advantages of monozygous twins over unrelated cows was shown: (*a*) by the close general correspondence of the within-twin-set results as compared with the between-twin-set results throughout the period of the trial; (*b*) by the similarity in the forms of the curves for the different sets of twins; (*c*) by the marked similarity of the reaction of the cows of a twin set to change in environmental conditions—this applied particularly for butterfat characteristics such as iodine value and refractive index; (*d*) by the 'twin efficiency values' for butterfat properties computed from the whole-lactation weighted means, viz. iodine value, 7·8; saponification value, 3·4; Reichert value, 5·5; carotene content, 7·8; vitamin A content, 1·7; and vitamin A potency, 2·5.

4. There were wide differences in the degree of approach to 'identicality' with different sets of twins. The cows in two of the sets showed a consistently close within-twin-set relationship for all characteristics studied.

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# 705. A COMPARISON OF DRIED SKIM-MILK AND WHITE FISH MEAL AS PROTEIN SUPPLEMENTS FOR FATTENING PIGS

## II. ON ALL-MEAL FEEDING UNDER COMMERCIAL CONDITIONS

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(With 1 Figure)

The experiment reported here was in essential details the same as that published earlier by Barber, Braude & Mitchell (1), except that the latter was carried out in an experimental piggery with individually fed, pure-bred, virus-pneumonia-free Large White pigs, whereas the present trial was done under commercial conditions with a variety of breeds and crosses and with group feeding. The object of the experiment was to obtain further information under these conditions on the comparative value of dried skim-milk and white fish meal as protein supplements for fattening pigs fed on all-meal diets.

### EXPERIMENTAL

#### (1) *Treatments*

There were four treatments (see Table 1):

(i) Meal containing 10% white fish meal, reduced to 7% from 130 lb. live weight.

(ii) Fish meal in (i) replaced by 10 and 7% dried skim-milk respectively (i.e. weight for weight basis).

(iii) Fish meal in (i) replaced by 15 and 10½% dried skim-milk respectively, with appropriate adjustments in the barley meal percentages.

(iv) Fish meal in (i) replaced by 20 and 14% of dried skim-milk respectively, with appropriate adjustments in the barley meal percentages (i.e. on a protein basis).

#### (2) *Diets*

The composition of the meal mixtures used is given in Table 1, together with their calculated crude protein* and starch equivalent values. These meal mixtures were identical with those used by Barber *et al.* (1), with the one exception that 3% of lucerne meal was included in each diet (replacing an equal weight of fine millers offal) as a source of vitamin A.

In Table 2 the percentage crude protein, calcium, phosphorus and salt in the white fish meal and dried skim-milk are shown. It is seen that two batches of both ingredients

* Using the figures in Table 2 for the white fish meal and dried skim-milk (first batches), and figures given by Woodman (2) for the other ingredients.

were used for the experiment. The composition of the meal mixtures and all analyses given in Table 1 are based on the figures for the first batches of these materials, the differences in composition between the first and second batches of both the white fish meal and the dried skim-milk being relatively so small that it was considered unnecessary to make any adjustments to the rations.

Table 1. *Percentage composition of meals used in the four treatments, together with calculated crude protein and starch equivalent values*

| Treatment | 1 | | 2 | | 3 | | 4 | |
|---|---|---|---|---|---|---|---|---|
| | To 130 lb. live weight | 130 lb.–slaughter | To 130 lb. live weight | 130 lb.–slaughter | To 130 lb. live weight | 130 lb.–slaughter | To 130 lb. live weight | 130 lb.–slaughter |
| Fine millers offal | 35 | 25 | 35 | 25 | 35 | 25 | 35 | 25 |
| Barley meal | 52 | 65 | 50½ | 64 | 45¾ | 60½ | 41 | 57¼ |
| Lucerne meal | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| White fish meal | 10 | 7 | — | — | — | — | — | — |
| Dried skim-milk | — | — | 10 | 7 | 15 | 10½ | 20 | 14 |
| Steamed bone flour | — | — | 1½ | 1 | 1¼ | 1 | 1 | ¾ |
| Aurofac 2A* | 2½ lb./2000 lb. in all meals | | | | | | | |
| Crude protein (%) | 17·7 | 15·5 | 14·9 | 13·5 | 16·4 | 14·5 | 17·8 | 15·5 |
| Starch equivalent | 63·8 | 65·8 | 65·2 | 66·8 | 66·0 | 67·2 | 66·7 | 67·8 |
| Starch equivalent / Crude protein ratio | 3·60 | 4·25 | 4·38 | 4·95 | 4·02 | 4·63 | 3·75 | 4·37 |

* Cyanamid of Great Britain Ltd., containing 3·6 g. chlortetracycline/lb.

Table 2. *Percentage crude protein, calcium, phosphorus and salt in the two batches of white fish meals and dried skim-milks used*

| | White fish meal | | Dried skim-milk | |
|---|---|---|---|---|
| | 1st batch | 2nd batch | 1st batch | 2nd batch |
| Crude protein | 66·1 | 62·9 | 38·4 | 35·2 |
| Calcium | 6·04 | 7·04 | 1·30 | 1·26 |
| Phosphorus | 3·41 | 3·95 | 1·04 | 0·98 |
| Sodium chloride | 1·62 | 1·89 | 1·86 | 1·70 |

### (3) *Pigs*

Details of the pigs used, their pre-experimental management, breeds, selection for experiment and housing were all identical with those described in detail by Braude, Clarke, Mitchell, Cray, Franke & Sedgwick (3). The treatments were allocated to the pens, each containing nine group-fed pigs, according to a definite pattern, the five pens of pigs on each of treatments (i) and (ii) being situated on the left side of the fattening house and those on treatments (iii) and (iv) on the right side of the house.

The first replicate of four pens of nine pigs each was selected on 14 October 1954. Thereafter, at weekly intervals, a further replicate was selected and put into the experimental house, the fifth and final replicate being selected on 11 November 1954.

### (4) *Pre-experimental treatment of pigs*

For the first week after being put in the experimental pens, all pigs continued to be fed on the proprietary creep pellets that they had received during suckling, plus unrestricted whey.

On the first day of the second week, whey feeding was stopped, only water *ad lib.* being supplied. At the same time a gradual change from creep pellets to the respective experimental meals was started so that by the fourth day of this week, only the meals were available. The experiment proper thus began when the age of the animals was 10 weeks (±3 days), that is, 2 weeks after the pigs had first been brought into the experimental pens.

### (5) *Records*

The pigs were individually weighed in the afternoon every 14 days, until they approached slaughter weight, when weighing was done once weekly. The pigs were fed once daily in the morning, all meal given being recorded. Whereas in the earlier experiment of Barber *et al.* (1), the pigs were fed with wet meal twice daily and according to a scale based on live weight, in the present experiment the meal was given dry and *ad lib.* up to a maximum of 6½ lb./pig/day. Water was provided *ad lib.* in separate troughs. Every fortnight, on the same day as the weighings were made, refusals of meal were weighed, so that records of meal consumption by each pen of pigs were obtained fortnightly throughout the experiment. The method described by Braude *et al.* (3) was used for estimating and allowing for the food consumed by pigs that either died or had to be taken off the experiment for various reasons that are discussed later.

### (6) *Carcass grading*

All details of the slaughter arrangements and carcass measurements taken were the same as those described by Braude *et al.* (3).

### (7) *Biometrical methods*

Means for each variable were calculated for each pen and subjected to the usual analysis of variance for a randomized block design, ignoring the fact that treatments were not allocated at random to available pen positions. In these analyses adjustments were not made for variation in either initial live weight or cold dead weight (see Barber, Braude & Mitchell (4)).

### (8) *Health of pigs*

During the course of the experiment eleven of the total of 180 pigs, or approximately 6%, either died or had to be taken off the experiment, the reasons for their removal being given in Table 3.

Table 3. *Details of pigs that died or were taken off the experiment*

| Treatment no. | No. of pigs involved | Cause of death or removal from experiment |
|---|---|---|
| 1 | 4 | Mange<br>Acute lameness<br>Died—pneumonia<br>Pneumonia |
| 2 | 4 | Mange (2)<br>Bowel oedema<br>Rupture |
| 3 | 1 | Pneumonia |
| 4 | 2 | Acute lameness<br>Enteritis |

Coughing, characteristic of virus pneumonia, was fairly widespread in the pigs. The same measures to control the secondary complications of this condition as were described by Braude *et al.* (3) were applied, and as a result a reasonably satisfactory over-all rate of growth was maintained by most of the pigs.

Several pigs in a number of pens became infected with parasitic mange; appropriate treatment with benzene hexachloride satisfactorily cleared up the trouble except in the few pigs which were withdrawn from the experiment.

## RESULTS

The mean results for live-weight gain, efficiency of food conversion and carcass measurements, together with the appropriate standard errors and levels of statistical significance are given in Table 4. The commercial grading results for the pigs are summarized in Table 5, while in Table 6 the average consumption of meal per pig per day over successive 14-day periods during the experiment, is given.

Table 4. *Treatments, mean live-weight gain, efficiency of food conversion and carcass measurements of pigs during a fattening period from approximately* 10 *weeks of age to a mean live weight of* 204 lb.

| | Treatment | | | | Differences between two means required for significance. Probability level | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | | | | |
| Protein supplement: | | | | | | | | |
| Up to 130 lb. live weight | 10% White fish meal | 10% Dried skim-milk | 15% Dried skim-milk | 20% Dried skim-milk | | | | |
| 130 lb.—slaughter | 7% | 7% | 10½% | 14% | 5% | 1% | 0·1% | Standard error |
| No. of pigs | 41 | 41 | 44 | 43 | — | — | — | — |
| Daily gain (lb.) | 1·12 | 1·23 | 1·27 | 1·27 | 0·05 | 0·07 | 0·10 | 0·016 |
| Efficiency of food utilization (lb. meal/lb. gain) | 4·38 | 4·35 | 4·25 | 4·27 | 0·12 | 0·17 | — | 0·040 |
| Carcass length (mm.) | 755·2 | 750·4 | 755·7 | 760·3 | 7·3* | 10·2 | — | 2·36 |
| Shoulder fat (mm.) | 50·4 | 51·5 | 50·0 | 50·6 | 2·16* | — | — | 0·702 |
| Mid-back fat (mm.) | 22·4 | 23·6 | 22·8 | 22·8 | 1·37* | — | — | 0·445 |
| Loin fat (mm.) 1 | 32·8 | 35·0 | 33·9 | 34·6 | 1·42 | 1·99 | 2·82 | 0·461 |
| 2 | 28·1 | 31·9 | 30·3 | 31·3 | 1·52 | 2·13 | 3·01 | 0·493 |
| 3 | 35·0 | 38·0 | 36·8 | 37·9 | 1·58 | 2·22 | 3·13 | 0·513 |
| Belly thickness (mm.) 1 | 34·0 | 35·1 | 34·8 | 35·2 | 1·58* | — | — | 0·513 |
| 2 | 31·2 | 32·9 | 31·7 | 32·3 | 1·53* | 2·15 | — | 0·497 |
| 3 | 31·1 | 31·8 | 32·5 | 31·4 | 2·28* | — | — | 0·740 |
| Eye muscle (mm.) Depth | 38·5 | 37·5 | 38·2 | 38·0 | 1·32* | — | — | 0·428 |
| Breadth | 87·2 | 85·2 | 86·5 | 85·7 | 2·75* | — | — | 0·891 |

* Mean square for treatment differences not significant at 5% level.

### (1) *Live-weight gain*

There was no significant difference in the rate of growth of the pigs receiving the three different levels of dried skim-milk, although the mean figures indicate that the 15 and 20% levels (treatments 3 and 4) were slightly better than the 10% level (treatment 2). All three groups receiving dried skim-milk grew at a significantly faster rate than the control pigs that had 10% white fish meal as the protein supplement in their diet ($P < 0{\cdot}001$).

(2) *Efficiency of food utilization*

There was a tendency for the pigs fed dried skim-milk to utilize their food somewhat more efficiently than the fish-meal-fed animals, although only for treatments 3 and 1 was the treatment difference statistically significant (treatment 3 < treatment 1, $P < 0{\cdot}05$).

(3) *Carcass measurements*

(i) *Length*

There was a tendency for an increase in carcass length with increasing levels of dried skim-milk in the ration. Differences between pigs on dried skim-milk and those on white fish meal were not significant.

(ii) *Back fat*

There were only small differences in either shoulder or mid-back fat thicknesses between any of the treatments and none of them was significant.

The pigs receiving each of the levels of dried skim-milk had a thicker loin fat at all three places than the fish meal controls, and most of these differences were significant at the 5% level or above.

Within the three groups receiving dried skim-milk, differences in loin fat thickness were smaller and in the main not significant.

(iii) *Belly thickness*

Treatment differences in belly thickness were small but there was a tendency towards a slight increase in belly thickness at all three positions as a result of replacing white fish meal by dried skim-milk in the ration.

(iv) *Eye muscle*

Treatment differences in both eye muscle depth and breadth were small and none of them was significant.

(4) *Commercial grading results*

The results in Table 5 show that the final grading of the control pigs on treatment 1 was appreciably better than that of the pigs receiving dried skim-milk on treatments 2–4, the better grading of the pigs on treatment 1 on the loin being particularly apparent.

Table 5. *Commercial grading* results of pigs*

| | | Final grade | | | | | | | | | | Pigs grading A on the loin | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. of pigs | Triple A | | A | | B | | C | | F | | | |
| Treatment | | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % |
| 1 | 41 | 21 | 51·2 | 26 | 63·4 | 9 | 22·0 | 6 | 14·6 | — | — | 25 | 61·0 |
| 2 | 41 | 11 | 26·8 | 18 | 43·9 | 13 | 31·7 | 9 | 22·0 | 1 | 2·4 | 16 | 39·0 |
| 3 | 44 | 13 | 29·5 | 22 | 50·0 | 18 | 40·9 | 4 | 9·1 | — | — | 18 | 40·9 |
| 4 | 43 | 14 | 32·5 | 20 | 46·5 | 14 | 32·5 | 7 | 16·3 | 2 | 4·7 | 17 | 39·5 |

* As in force when the pigs were slaughtered.

Within the groups receiving the three levels of dried skim-milk, those on treatment 3 (15% skim-milk) gave slightly better over-all grading results.

(5) *Pattern of food consumption during the experiment*

In considering the results in Table 6, it must be remembered that some pigs from all treatments were ready for slaughter by the 21st–23rd weeks of life, and that in consequence from this stage onwards the mean figures in the table refer to continually decreasing numbers of animals.

Table 6. *Mean consumption of meal (lb./pig/day) over 14-day periods during the experiment (except for the first period which was 7 days only)*

| | Treatment no. | | | |
|---|---|---|---|---|
| Protein supplement: | 1 | 2 | 3 | 4 |
| Up to 130 lb. live weight | 10% White fish meal | 10% Dried skim-milk | 15% Dried skim-milk | 20% Dried skim-milk |
| 130 lb.—slaughter | 7% White fish meal | 7% Dried skim-milk | 10½% Dried skim-milk | 14% Dried skim-milk |
| Approximate age of pigs (weeks) | | | | |
| 10–11 | 2·7 | 3·0 | 3·0 | 3·0 |
| 11–13 | 2·8 | 3·5 | 3·4 | 3·3 |
| 13–15 | 3·2 | 4·1 | 4·1 | 4·0 |
| 15–17 | 3·7 | 4·6 | 5·0 | 5·1 |
| 17–19 | 4·4 | 5·3 | 5·7 | 5·7 |
| 19–21 | 5·0 | 5·9 | 6·2 | 6·3 |
| 21–23* | 5·8 | 6·5 | 6·4 | 6·5 |
| 23–25 | 6·3 | 6·5 | 6·4 | 6·4 |
| 25–27 | 6·5 | 6·4 | 6·5 | 6·5 |
| 27–29 | 6·5 | 6·4 | 6·4 | 6·0 |
| 29–31 | 6·5 | 6·3 | 6·2 | 5·8 |
| 31–33 | 6·4 | 6·2 | 6·2 | 5·5 |
| 33–35 | 6·4 | 6·5 | 6·0 | 5·2 |

* From this stage onwards pigs were sent for slaughter, consequently the figures refer to a continually changing number of animals.

It is seen that the level of meal consumption, as related to age, of the pigs receiving dried skim-milk in their ration was higher than that of the control animals right from the first week of the experiment, and that they were eating the maximum daily allowance of 6½ lb. of meal by the 21st–23rd weeks, whereas the control pigs did not reach this maximum until 4 weeks later.

## DISCUSSION

Contrary to the results published earlier by Barber *et al.* (1), in which replacement of a supplement of 10% reduced to 7% of white fish meal by an equal weight of dried skim-milk had no significant effect on the rate of growth of the pigs, in the present experiment this same replacement resulted in a significantly faster growth rate (treatment 2 > 1 at 0·1% level of significance). No significant effect on food conversion efficiency was observed between these two treatments in either experiment, however.

As in the previous experiment, the mean results in the present work (Table 4) suggest a trend towards slightly improved growth rate and efficiency of food utilization when the amount of dried skim-milk in the diet was increased to 15%. Contrary to the earlier results, however, no further improvement was apparent when the dried skim-milk content was raised to 20%.

In considering the contradictory results obtained in the two experiments when white fish meal was replaced by dried skim-milk on a weight for weight basis, the results in Fig. 1, showing the mean growth curves of the pigs for the pre-experimental period of 2 weeks and for the first 11 weeks of the experimental period (when the first pig went to slaughter), are of interest. It is seen that in the pre-experimental period the growth rates of the four groups of pigs did not substantially differ and they were at a reasonable level for pigs of that age. In the first week of the experiment, however, whereas all the pigs

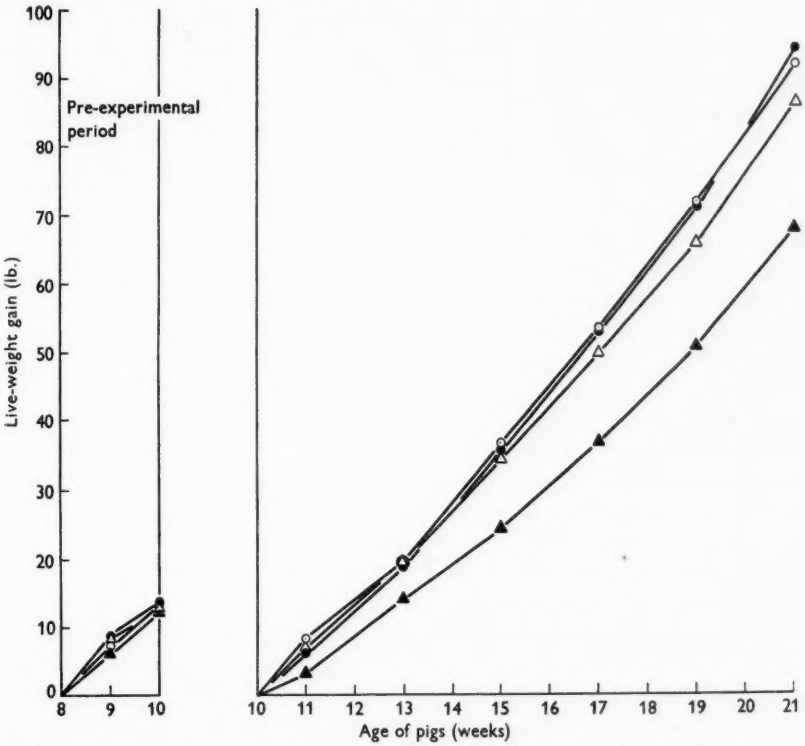


Fig. 1. Growth curves of pigs during the pre-experimental period of 2 weeks and during the first 11 weeks of the experiment. ▲—▲, treatment 1 (10% white fish meal); △—△, treatment 2 (10% dried skim-milk); ●—●, treatment 3 (15% dried skim-milk); ○—○, treatment 4 (20% dried skim-milk).

receiving dried skim-milk in the diet continued to gain approximately 1 lb./day, the control pigs grew at only about half that rate. The same over-all picture was apparent in each of the five individual replicates and indicates that the control pigs suffered a definite set-back at this time.

This conclusion is supported by the results in Table 6, which show the higher level of food consumption, as related to age, of the skim-milk fed pigs, compared with the control animals, right from the first week of the experiment. Since all pigs had received whey as part of their diet in early life, one can speculate that this set-back in the fish-meal controls was at least in part, the result of the change to a diet containing no milk products. However, since the growth curves in Fig. 1 show that the three groups of animals receiving

dried skim-milk continued to grow faster than the controls right through the 11-week period, it would seem unlikely that the final differences in rate of growth were the result of the initial set-back alone.

A further striking difference between the results obtained in the present experiment and those of Barber *et al.* (1) is in the absolute performance of the animals regardless of treatment. In the former experiment the average daily gains and food consumed/lb. gain ranged from 1·42 to 1·54 and from 2·99 to 3·20 respectively, whereas in the present experiment the corresponding figures were 1·12 to 1·27 and 4·25 to 4·38.

There are many possible reasons for these differences, such as the presence of virus pneumonia in the pigs in the present experiment, whereas Barber *et al.* (1) used virus-pneumonia-free stock, temperature differences in the buildings and so on, but no useful purpose would be served here by speculating as to which are likely to be the more important factors involved. The general observation can be made, however, that where such marked differences in general performance occur, differences in treatment response are not to be unexpected.

One difference between the two experiments which, however, should be mentioned as having an obvious effect on the comparative performance of the pigs, concerns the method of feeding. In the present trial the pigs were group fed *ad lib.* once daily up to a daily maximum of 6½ lb./head, whereas in the earlier experiment the pigs were fed individually twice daily according to a scale based on live weight, up to a maximum of 6½ lb./head.

The effect on food consumption, growth, efficiency of food conversion and carcass quality of 'semi *ad lib.*'* feeding compared with feeding to a pre-determined scale, both feeding systems having the same maximum daily meal allowance, was demonstrated by Barber *et al.* (4), and there can be no doubt that the difference in method of feeding used in the present experiment and that of Barber *et al.* (1), was responsible in part for the differences in the absolute performance of the pigs in the two experiments.

Under the conditions of the present experiment, the results strongly suggest that as a protein supplement dried skim-milk is of greater value to the pig than white fish meal, although the two points that were discussed in detail by Barber *et al.* (1), namely, that the level of protein in the control diet might be too generous, and that the dried skim-milk diets had a higher energy value than the fish-meal control diet, apply equally in the present experiment. Although the differences between treatments 2 and 3 in both growth rate and food utilization did not quite reach the recognized level of statistical significance, the over-all results indicate that in general a slightly better performance can be expected when the level of dried skim-milk in the ration is raised from 10 to 15%, which agrees with the conclusions of Barber *et al.* (1).

The general tendency in the carcass measurement results obtained by Barber *et al.* (1) for the pigs fed dried skim-milk to have slightly thicker back fats than the control pigs, was repeated in the present experiment. Contrary to the earlier results, however, these differences were clearly reflected in the commercial grading results shown in Table 5, the poorer grading on the loin of the pigs fed dried skim-milk being particularly apparent. The suggestion by Barber *et al.* (1), that such differences in back fat thicknesses, while not being reflected in the commercial grading results in pigs with a high grading potential,

* Defined as: pigs given as much food as they can clear up in 30 min. at two feeds daily up to some maximum daily allowance.

might be so in pigs having a lower grading potential, appears, therefore, to have been borne out by these results. Clearly, under conditions where the general level of grading in a herd is low, this possible adverse effect on the grading of replacing fish meal by dried skim-milk in the ration is an important factor to consider in deciding which protein supplement should be used.

The agreement in both experiments for the skim-milk fed pigs to have thicker bellies and a smaller breadth of eye muscle suggests that these results are probably true treatment effects in spite of the fact that within each experiment the differences were in the main not statistically significant at the recognized level. The practical importance of the relatively small differences that occurred is, however, questionable.

One final point on the grading results should be mentioned. As was pointed out by Braude *et al.* (3), it appears that with the breeds and strains of pigs used in these trials, a maximum daily meal allowance of 6½ lb./pig is too high and results in over-fat carcasses. An experiment to determine the optimum level of all-meal feeding under the conditions of these trials will be the subject of a paper to be published shortly.

## SUMMARY

1. Results are reported of an experiment carried out under commercial conditions to obtain information on the comparative value of dried skim-milk and white fish meal as protein supplements for fattening pigs.

2. There were four treatments. Control pigs (i) received a meal mixture containing 10% white fish meal, reduced to 7% from 130 lb. live weight. In the other experimental groups, the fish meal was replaced by dried skim-milk, (ii) on a weight for weight basis, (iv) on a protein basis and (iii) intermediate between (ii) and (iv). All pigs were fed the meal dry and *ad lib.* up to a maximum of 6½ lb./head daily. Unrestricted water was available.

There were five pens of nine group-fed pigs on each treatment, involving a total of 180 pigs. The pigs were on experiment from approximately 10 weeks of age to bacon weight. Comprehensive carcass measurements were made on all the pigs.

3. The results showed that a significantly better rate of growth was obtained when the white fish meal was replaced by dried skim-milk on a weight for weight basis. Increasing the percentage of skim-milk in the diet up to 15% resulted in a slightly better performance of the pigs, but no further improvement was obtained by raising the level of skim-milk to 20%. These results were discussed in relation to an apparent set-back that the control pigs suffered at the start of the experimental period.

4. The inclusion of dried skim-milk in the diet had an adverse effect on the carcass grading, particularly in so far as it resulted in a thicker layer of back fat over the loin as compared with that in the control pigs.

5. There was also some indication that the carcasses from the animals given dried skim-milk had slightly thicker bellies and a smaller breadth of eye muscle than the carcasses of the control animals that received fish meal.

6. The results have been compared with those of an earlier trial, which was in most details the same as that reported here, the main difference being that in the earlier experiment individual feeding and one breed of pig were used.

We thank the Directors of Messrs C. & G. Prideaux, Ltd., Bacon Curers, Motcombe, Shaftesbury, Dorset, for permission to take carcass measurements on all the experimental pigs, and we are greatly indebted to Mr K. Lawrence who most conscientiously took these measurements.

We are grateful also to Miss J. N. King for her help with the statistical computations.

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# 706. LONG SURVIVAL OF A STREPTOCOCCAL BACTERIOPHAGE

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Bacteriophage active against a lactic streptococcus was first isolated in this laboratory in February 1935. In May of the same year some batches of phage for *Streptococcus cremoris* strain HP were prepared by lysing the bacteria in skim-milk culture and preparing a Seitz-filtered whey as described by Whitehead & Cox (1). At that time it was not the practice to adjust the reaction of phage filtrates and the pH of the batches prepared in 1935 was not recorded. It was probably between 5·2 and 6·1 (Hunter & Whitehead (2)). Samples of four different preparations of the phage were filled into sterile tubes and sealed off in a blow-pipe flame. Two of the samples, sealed on 24 May 1935, had titres of $10^7$ (as determined by spotting of standard loops of dilutions on a bacterial mat) and the other two, sealed on 16 July 1935 had titres of $10^6$. The sealed tubes were stored in the refrigerator at 4–7° C.

In March 1951 the tubes were opened and the activities of the phage preparations against strain HP were investigated. None of the samples gave plaques on a bacterial mat on an agar plate even when undiluted. However, when 0·25 ml. of phage preparation was added to a 9 ml. culture of HP in skim-milk at 22° C., the culture was lysed overnight by three of the preparations. From the lysed culture normal phage for HP was recovered. The residue of each of the three active phage preparations (amounting to between 1 and 2 ml.) was put into a fresh sterile tube sealed with a bark cork sterilized by a dip in hot wax. The tubes were replaced in the refrigerator.

In September 1957, the preparations were retested, as before. Two of the three still showed activity when 0·5 ml. of phage was added to a 9 ml. culture of HP in skim-milk. The third sample no longer had any action on the culture, even when 1 ml. was used (no more of the sample was available). The pH values of the two active preparations were 5·65 and 5·75. A fresh phage preparation made from one of the samples was shown to have the same range of action on stock cultures as the stock HP phage.

These results indicate therefore that under the conditions described, the streptococcal phage survived for more than 22 years. During the first 16 years the numbers of phage particles per ml. decreased from more than 10 millions to less than 250 (the standard loop spotted on a plate holds 0·004 ml.). The decrease in numbers of particles between 1951 and 1957 was, however, very slight, and probably the phage would have been recoverable from larger volumes of the samples for several more years.

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637.127.1:547.953.2

# 707. PHOSPHOLIPIDS IN NEW ZEALAND DAIRY PRODUCTS

## I. THE ESTIMATION OF THE TOTAL PHOSPHOLIPIDS AND OF LECITHIN, CEPHALIN AND SPHINGOMYELIN IN BUTTER

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The results reported in the literature for phospholipid content of milk and its products, including butter, show a wide range of variation (1,2). It seems likely, however, that much of this variation is due to differences in the method of extraction and, in particular, to use of different solvents. The assumption of many workers that in the Röse-Gottlieb method of fat estimation in milk and its products the solvent extracts the phospholipids quantitatively is not supported by adequate experimental evidence.

Identification of the individual phospholipids in milk and its products was undertaken by several of the earlier workers, all of whom reported the presence of the monoamino phosphatides lecithin and cephalin (3,4,5,6). Some of these investigators detected also the presence of a diamino phospholipid (5,6), which later was identified as sphingomyelin (7). Crane & Horrall (8) estimated the total phospholipid and the lecithin plus sphingomyelin contents of several dairy products including milk and butter. Baliga & Basu estimated the contents of each of the three phospholipids in a number of milks (9) and in a few samples of cream and creamery butter (10). Apart from this there is little information available on the proportion of the different phospholipids found in milk, cream and butter.

The present paper describes a method, based on a variation of the Röse-Gottlieb method, for extraction of the phospholipids from butter, and methods for the routine estimation of total and of individual phospholipids in the extract. Alternative methods of extraction also are described, and results are compared with results obtained by the modified Röse-Gottlieb procedure.

### EXPERIMENTAL

#### *Preparation of serum from butter*

When butter is carefully melted at a comparatively low temperature (under 50° C.) only a trace of phosphorus is found in the separated butterfat (11,12,13,14). For estimation of phospholipids, therefore, only butter serum was required.

(*a*) *Serum for extraction of phospholipids by various solvents.* Butter (70–80 g.) was melted at 40–45° C. in a centrifuge tube and the serum was separated from the fat by centrifuging. The fat was drawn off and the residue was cooled in a refrigerator to solidify the last traces of fat. The fat-free serum (serum A) was then poured off into a clean flask.

(*b*) *Serum for routine estimation of phospholipids.* Butter (11 g.) in a 15 ml. centrifuge tube was melted at 40–45° C. and the serum was separated from the fat by the method described above. After cooling the serum in the refrigerator 10 ml. of water were added, the diluted serum (serum B) was poured off into a small beaker and the last traces of fat were removed with strips of filter paper.

## EXTRACTION OF PHOSPHOLIPIDS FROM BUTTER SERUM AND ESTIMATION OF TOTAL AND OF INDIVIDUAL PHOSPHOLIPIDS IN THE EXTRACT

### (1) *Methods of extraction*

(*a*) *Direct extraction with ether-light petroleum mixture* (*modified Röse-Gottlieb method*). The method used for extraction and estimation of fat in milk was modified for butter serum. The procedure was as follows: Two ml. of serum A (see above) were weighed in a tared beaker, diluted with 10 ml. of water, and transferred to a Mojonnier tube (as used for the Mojonnier modification of the Röse-Gottlieb method) with 10 ml. of 95–96% ethanol. The total volume of serum B (see above), was transferred to a Mojonnier tube with 10 ml. of 95–96% ethanol.

The mixture from either serum A or serum B was extracted with ether and light petroleum (30 ml. of each) as in the routine method for estimation of fat in dairy products. For the second extraction 5 ml. of 95–96% ethanol and 35–40 ml. of the mixed solvents were added. The combined extracts were made finally to a volume of 100 ml. with ether and light petroleum.

Omission of the dilution of the serum with water led to low and inconsistent results for total phosphorus in the extract. Addition of ammonia (2 ml.) to the diluted serum (from salted or unsalted butter) before extraction, as specified in the standard Röse-Gottlieb procedure for milk, also led to low results. Ammonia is added to promote solution of the proteins and might be expected to have little effect on the extraction of the phospholipids from butter serum in comparison with the extraction from other dairy products in which part, at least, of the phospholipids is associated with the fat globule membrane. This was verified in experiments with milk, skim-milk and buttermilk (none of which were diluted with water before extraction) from all of which the amount of phospholipid extracted was greater after the addition of ammonia. However, in butter serum already diluted 1 to 5 with water the increase in pH after adding ammonia probably was much greater than in undiluted milk, skim-milk or buttermilk. Since lecithin and cephalin are hydrolysed under mild alkaline conditions it is possible that there was some loss of these phospholipids in ammoniacal butter serum solutions and that the recovery of total phospholipids was low. Thus in the present paper all results for phospholipids from ether-light petroleum extracts of butter serum refer to extractions in which addition of ammonia was omitted.

(*b*) *Direct extraction with ethanol-ether.* Methods described in the literature for the extraction of phospholipids from milk and its products include that proposed by Bloor originally for blood serum, using as solvent ethanol-ether in the ratio 3:1 (15,16). When applied to butter serum in the present investigation the method was as follows: 2 ml. of serum were run slowly from a pipette into 50 ml. of a mixture of ethanol (95%) and ether (3:1). The mixture was brought just to boiling in a hot water-bath, and was then cooled and filtered. The residue and filter-paper were washed with ethanol-ether and the combined extracts were brought to a total volume of 100 ml. with ethanol-ether.

(*c*) *Extraction of trichloroacetic acid* (*TCA*) *precipitate with various solvents.* A mixture of chloroform-methanol (1:1) has been used to extract quantitatively the phospholipids from freeze-dried blood and plasma (17). The same solvent was used by Haylock at this Institute to extract the phospholipids from freeze-dried butter serum (12). Haylock found, however, that added inorganic phosphate was also extracted. He therefore precipitated the proteins and phospholipids in butter serum with TCA, separated them by filtration

and extracted the precipitate with chloroform-methanol. His results for extracted phosphorus by this method were higher than those obtained by a modified Röse-Gottlieb method of extraction, but added inorganic phosphate was not present.

This preliminary precipitation with TCA before extraction was investigated by the present author. Solvents other than chloroform-methanol used for the extraction of the precipitate were ethanol-ether, benzene-ethanol and benzene-isopropanol. Other workers have found that benzene-ethanol extracts considerably more phosphorus from milk and milk products than does benzene-isopropanol (18,19,20). The procedure for the precipitation of the serum proteins and the subsequent extraction of the phospholipids was as follows:

Serum (5 ml.) was mixed with 20 ml. of water and 25 ml. of 25% TCA, and after 5 min. the mixture was filtered through a Whatman no. 50 fluted paper. The precipitate, after washing with a little water, was dried on the filter-paper in a vacuum oven at 35° C. The dried filter-paper and precipitate were transferred to a 200 ml. Kjeldahl flask and 150 ml. of solvent were added. The mixture, shaken at frequent intervals, was brought just to boiling in a hot water-bath, and then it was cooled and filtered through a no. 4 sintered glass crucible. The Kjeldahl flask and crucible were washed with 50–100 ml. of the same solvent and the two extracts were combined and made up to a total volume of 250 ml. The solvents were all of analytical reagent quality and mixtures were prepared as follows: ethanol-ether, 3:1; all other mixtures, 1:1.

(*d*) *Extraction of the colloidal-iron precipitate with benzene-ethanol.* Colloidal iron, which avoids the high acid concentration of TCA, has been used as a protein precipitant in the estimation of phospholipids in blood serum (21). It was found in the present investigation that butter serum proteins could be precipitated by this reagent. The precipitate was extracted with benzene-ethanol which, in the work on extraction of the TCA precipitate ((*c*) above), was found to filter more rapidly than any other of the solvents.

Details of the procedure for precipitation with colloidal iron and the subsequent extraction were as follows: colloidal iron solution (6 ml.) was added slowly with shaking to 5 ml. of butter serum diluted with 25 ml. of water. The mixture was filtered through a Whatman no. 50 fluted paper and washed with a solution of 1% magnesium sulphate. The paper and precipitate were dried in a vacuum oven at 35° C. and then transferred to a Kjeldahl flask. The method of extraction with benzene-ethanol (1:1) was the same as that described above for the TCA precipitate.

(*e*) *Direct extraction with chloroform-methanol.* Several workers have used chloroform-methanol, without a preliminary precipitation of proteins, to extract the phospholipids from brain tissue (22), blood serum (23) and reconstituted dried whole egg (24). These workers, however, all recommend washing the extract with water, which is said to eliminate non-phospholipid compounds and soluble choline (22,24). The extraction of butter serum with chloroform-methanol and washing the extract with water were investigated by the present author.

The method of extraction was as follows: serum (2 ml.) was added to 20 ml. of methanol in a Squibb separating funnel. The mixture was well shaken after the addition of 20 ml. of chloroform and again after the addition of 8 ml. of water. The separation into two layers was completed by centrifuging and the lower solvent layer was run off. The residue was extracted with a further 25 ml. of chloroform and, after centrifuging, the chloroform layer was removed. The combined extracts were dried over sodium sulphate and their total volume made up to 100 ml. with chloroform-methanol (2:1).

(2) *Digestion of extracts and estimation of total phosphorus*

A measured volume (1 ml.) of extract was pipetted into a $6 \times \frac{3}{4}$ in. Pyrex test tube (25) (previously washed with chromic acid and rinsed with water) and the solvent evaporated on a water-bath. One ml. of 10 N sulphuric acid and 3 drops of 30% hydrogen peroxide were added and the mixture was heated carefully over a micro-burner on a micro-Kjeldahl stand until dense white fumes appeared (25,26). Further additions of hydrogen peroxide were often necessary to complete the digestion. Finally, the last traces of hydrogen peroxide were removed by heating after the addition of 0·3–0·5 ml. of water until white fumes appeared. The digest was cooled, 8·7 ml. of water, 0·7 ml. of 2·5% ammonium molybdate solution and 0·5 ml. of 1:2:4 aminonaphtholsulphonic acid reagent (prepared according to King (27)) were added, and the mixture shaken. The tube was placed in a boiling water-bath for 10 min.,* cooled in running water and the solution made up to 10 ml. with water. The intensity of colour was compared in the Beckman spectrophotometer at 830 mμ (28) with that of standards prepared from the same quantities of acid and reagents. Blank estimations on 1 ml. quantities of the solvent were included in each batch of samples digested.

(3) *Estimation of total nitrogen content of extracts*

The total nitrogen contents of the extracts from the TCA precipitate of unsalted butter serum (see Results, Table 1, N:P ratios) were estimated as follows: a small portion of the extract was digested with sulphuric acid and hydrogen peroxide as described above. The digest was neutralized to a pH between 4 and 8 and the intense blue colour given by the addition of phenol-hypochlorite-ammonia reagent in alkaline solution (32) was compared with colours of standard nitrogen solutions treated similarly. The results were only approximate as the method is unsuitable for micro-Kjeldahl digests (32).

The total nitrogen contents of all other extracts were estimated indirectly as the difference between the Kjeldahl nitrogen results for the serum or the protein precipitate before and after extraction.

(4) *Estimation of individual phospholipids*

(*a*) *A routine method for ether-light petroleum extracts.* The method used for routine estimation of lecithin, cephalin and sphingomyelin in ether-light petroleum extracts of butter serum was based on the procedures described by Hack for blood phospholipids (17) and by Johnson, McNabb & Rossiter for brain phospholipids (33). A portion of the extract, freed from the solvent mixture by evaporation, was hydrolysed with potassium hydroxide. Phosphorus from lecithin and cephalin, and choline from lecithin were liberated quantitatively by this treatment, but sphingomyelin was not affected. Choline was estimated as the reineckate according to Glick's method (34), except that the choline reineckate salt after precipitation was not filtered—instead, the mixture was centrifuged and the supernatant liquid drawn off. The reineckate salt after washing with *n*-butanol (35) was dissolved in acetone and was estimated by measuring the intensity of absorption at 329 mμ.†

* Several workers, using aminonaphtholsulphonic acid as reducing agent, have found that heating in a boiling water-bath increases the intensity of colour very greatly (26, 28, 29, 30). Most authors, however, appear to be unaware that Fiske & Subbarow in their original paper (31) observed that the blue colour could be intensified by heating.

† Pure choline reineckate showed a slightly greater absorption at 329 mμ than at 327 mμ—the wavelength used by Winzler & Meserve (36) for this salt.

The final procedure was as follows:

(i) *Alkaline hydrolysis.* From a portion (25 ml.) of the ether-light petroleum extract of butter serum the solvents were removed by evaporation under reduced pressure in a water-bath at 50° C. To the residue in the flask 5 ml. N-KOH were added and the flask was stoppered and placed in an incubator at 37° C. for 24 hr. for completion of hydrolysis. A blank estimation on 5 ml. of N-KOH was included with each batch of samples hydrolysed.

(ii) *Estimation of soluble phosphorus.* One ml. of the hydrolysed solution in a test tube was acidified with 1 ml. of 1·5 N-HCl and 3 ml. of 0·5 M-trichloroacetic acid. After 1 hr. the mixture was filtered through a 7 cm. Whatman no. 50 paper. A measured volume (1 ml.) of the filtrate was taken for digestion and estimation of phosphorus as described under (2) above.

(iii) *Estimation of choline.* The remainder of the hydrolysed solution was acidified with 0·24 ml. of glacial acetic acid and filtered through a 7 cm. no. 42 Whatman paper into a 15 ml. centrifuge tube. The precipitate was washed twice with 2 ml. of water and the filtrate and washings were mixed. Two ml. of a 2% solution of ammonium reineckate in methanol were added and the mixture was left in the coldest part of the refrigerator for 1½–2 hr. to complete the precipitation of the choline salt. The tube was centrifuged at 2500 revolutions/min. for 5 min. and the supernatant liquid was carefully drawn off through a fine capillary. The precipitate was washed by vigorous shaking with 2 ml. of *n*-butanol, the mixture centrifuged and the butanol drawn off. The washing was repeated twice. Finally, 10 ml. of acetone were added and the choline salt was dissolved by shaking just prior to measuring the intensity of absorption at 329 mμ in the Beckman spectrophotometer.

(iv) *Calculation of results for partition of phosphorus among the individual phospholipids.* As the lecithin molecule contains one choline and one phosphate group, the content of lecithin phosphorus in butter serum was calculated from the choline content. The contents of sphingomyelin phosphorus and of cephalin phosphorus were calculated as follows:

$$\text{total P} - \text{soluble P} = \text{sphingomyelin P},$$

$$\text{soluble P} - \text{lecithin P} = \text{cephalin P}.$$

(*b*) *Method used when comparing results from mixed ether extracts with results from extracts with other solvents.* Results for cephalin and sphingomyelin estimated by the above method would be seriously in error for extracts containing non-phospholipid phosphorus compounds. Hence, for comparison of lecithin, cephalin and sphingomyelin contents of various extracts from the same butter serum, as in Table 2 (see later), the sphingomyelin P content was calculated from its content of choline. Sphingomyelin choline was estimated as the difference between results for choline (*a*) after hydrolysis with baryta which liberates choline from both lecithin and sphingomyelin and (*b*) after hydrolysis with potassium hydroxide (see above) which liberates choline from lecithin alone. The difference in the results for total P content of the extract and for lecithin plus sphingomyelin P contents is reported as 'cephalin' P. This result will include phosphorus not only from cephalin itself but from non-phospholipid compounds.

The method for hydrolysis with baryta was as follows: From 25 ml. of the extract the solvents were removed on a water-bath at 50° C. under reduced pressure. The residue was refluxed with 10 ml. of 0·25 N baryta (37) for 2 hr. It was then cooled and acidified with

0·14 ml. of acetic acid. The mixture was filtered, the residue was washed twice with 4 ml. of water and the filtrate and washings were made up to a total volume of 18 ml. The choline content of 9 ml. of this liquid was estimated by the method already described ((4), (iii) above).

(5) *Calculation of results*

Factors for the conversion of lecithin and cephalin P results to percentage of phospholipids were calculated from Shorland's analysis (38) of the fatty acids in phospholipids from New Zealand butter serum. The conversion factors were: lecithin 25·5, cephalin* 24·4. The factor for sphingomyelin P was 26·8 (39) since lignoceric acid is the predominating acid.

A factor for the conversion of total phosphorus content of butter serum to percentage of total phospholipids was calculated from the above figures and from the average proportions of each component phospholipid in butter (see part II of this series (40)). This factor, 25·5, is intermediate between those of 25·00 and 25·94 suggested by Holm *et al.* (1) and by Horrall (41) respectively.

## RESULTS

### *Extraction of butter serum with ether-light petroleum mixture*

The ether-light petroleum mixture in the modified Röse-Gottlieb method of extraction, as described above, was found not to extract inorganic P from butter serum, thus confirming the findings of earlier workers for milk (42) and cream (43). The solvent mixture apparently did not extract any non-phospholipid organic phosphorus compounds since the N:P ratio of the extracts (see Tables 1 and 2) was similar to that for a mixture of lecithin, cephalin and sphingomyelin, i.e. between 1 and 2. Thus it may be assumed that the total P content was a true index of the phospholipid content of the extract.

Table 1. *The effect of various solvents on the amount of phosphorus extracted from butter serum from unsalted and salted butters*

| Method of extraction | Extracted P in salt-free serum (mg./100 g.) | | N:P ratio in unsalted serum extract |
|---|---|---|---|
| | Unsalted | Salted | |
| Ether-light petroleum extraction of serum | 38·2 | 38·3 | 0·9 |
| Ethanol-ether extraction of serum | 45·7 | 42·6 | — |
| Ethanol-ether extraction of TCA precipitate | 41·3 | 38·2 | 2·3 |
| Chloroform-methanol extraction of TCA precipitate | 47·5 | 45·3 | 4·0 |
| Benzene-ethanol extraction of TCA precipitate | 43·4 | 43·1 | 4·2 |
| Benzene-isopropanol extraction of TCA precipitate | 34·7 | 37·8 | 1·4 |

### *Comparison of the results from ether-light petroleum extracts of butter serum with results from other methods of extraction*

(1) *Direct extraction with ethanol-ether*

In a trial with ethanol-ether (3:1), it was found that the solvent extracted considerably more phosphorus from sera from unsalted butter and from the same butter salted to 1·6% than did the ether-light petroleum mixture (see Table 1). The ethanol-ether solvent,

* The assumption was made that cephalin consists of equal quantities of phosphatidyl serine and phosphatidyl ethanolamine.

Table 2. *The effect of various solvents on the amounts of total phosphorus and of lecithin, cephalin and sphingomyelin phosphorus extracted from butter sera*

| Sample | Method of extraction | Total extracted P in serum (mg./100 g.) | N:P ratio of extract | Extracted P in serum (mg./100 g.) | | |
|---|---|---|---|---|---|---|
| | | | | Lecithin P | Sphingomyelin P | Cephalin P |
| A | Ether-light petroleum extraction of serum | 24·6 | 1·3 | 7·3 | 8·8 | 8·5 |
| | Ethanol-ether extraction of TCA precipitate | 26·3 | 3·5 | 7·8 | 9·1 | 9·4 |
| | Chloroform-methanol extraction of TCA precipitate | 29·3 | 10·3 | 8·1 | 7·3 | 13·9 |
| | Benzene-ethanol extraction of TCA precipitate | 25·8 | 4·3 | 7·9 | 8·9 | 9·0 |
| | Benzene-isopropanol extraction of TCA precipitate | 25·6 | 3·9 | 7·8 | 8·4 | 9·4 |
| B | Ether-light petroleum extraction of serum | 27·0 | — | 7·4 | 7·5 | 12·1 |
| | Chloroform-methanol extraction of serum | 27·7 | 3·6 | 7·3 | 8·6 | 11·8 |
| | Benzene-ethanol extraction of colloidal iron precipitate | 26·5 | 2·1 | 8·1 | 6·7 | 11·7 |
| C | Ether-light petroleum extraction of serum | 26·2 | 2·4 | 7·1 | 7·2 | 11·9 |
| | Chloroform-methanol extraction of serum | 26·6 | 4·0 | 6·9 | 6·7 | 13·0 |

however, has been found by other workers to extract from milk relatively large quantities of inorganic phosphate (42) and of non-phospholipid P (44). Hence it appears likely that in the present investigation the increase in P content of the extract with this solvent compared with that obtained with the ether-light petroleum mixture was due to non-phospholipid P.

(2) *Extraction of TCA precipitate with various solvents*

Results are given in Table 1 for sera from unsalted butter and from the same butter salted to 1·6%.* The results show that, in comparison with the ether-light petroleum extracts of the butter sera: (i) chloroform-methanol and benzene-ethanol extracted more P from the precipitates from both salted and unsalted butter sera; (ii) ethanol-ether extracted more P from the TCA precipitate from the unsalted butter serum but the same amount from the precipitate from the salted butter serum; (iii) benzene-isopropanol extracted less P from the precipitates from both sera.

The high results obtained with some of the solvents could be due to a more efficient extraction of true phospholipids or to extraction of non-phospholipid P compounds. Results for the N:P ratios in the extracts from the unsalted butter serum are shown in Table 1. It will be seen that both chloroform-methanol and benzene-ethanol extracted appreciable quantities of nitrogen-containing compounds other than phospholipids. Hence it is possible that one or more of the component fractions of casein were being partially extracted by these solvents.

Results for the serum from a second sample of salted butter (sample A) are shown in Table 2. It will be seen that ethanol-ether, chloroform-methanol, benzene-ethanol and

* The TCA precipitates in this trial were not dried before extraction. In later trials the precipitate was dried at a low temperature in a vacuum oven.

benzene-isopropanol all extracted more P from the TCA precipitate than did the ether-light petroleum mixture from the serum itself. The N:P ratios, however, were greater than that of the ether-light petroleum extracts.

Results for contents of lecithin, cephalin and sphingomyelin P for each extract from sample A are included in Table 2. The higher values for total P contents of the extracts from the TCA precipitates, in comparison with values for the ether-light petroleum extract, were due, apparently, to more efficient extraction of the component phospholipids by three of the solvents, viz. ethanol-ether, benzene-ethanol and benzene-isopropanol. The 'cephalin' P content of the chloroform-methanol extract was much higher than that of any of the other extracts. Since the N:P ratio also was much higher it appears that compounds other than phospholipids were extracted by this solvent.

The solvent effect of chloroform-methanol on pure casein, prepared by the method of Linderstrom-Lang, which includes final washing of the casein with 96% ethanol and ether (45), was investigated. Small samples (0·125 g.) of casein were heated with chloroform-methanol or with ethanol-ether (3:1), using the same quantities of solvents and the same procedure as in the extraction of the TCA precipitates (see under 'Experimental'). The P content of the casein sample was 0·60%, and 6·7% of this P was found in the chloroform-methanol extract. The N:P ratio of the extract (which contained 1·8% of the N content of the casein) was 85·8 compared with 50·0 for the pure casein. The alcohol-ether extract contained only a trace of P, although the N present was equivalent to 3·6% of the casein N. The results support the earlier suggestion that casein in the TCA precipitate is partly soluble in chloroform-methanol.

(3) *Extraction of colloidal iron precipitate with benzene-ethanol*

The results from an extraction of the colloidal iron precipitate from the serum of a salted butter (sample B) are shown in Table 2. The amount of total phosphorus extracted was slightly less than that extracted from the serum by the ether-light petroleum mixture. However, results for lecithin, cephalin and sphingomyelin in both extracts agree fairly well. The N:P ratio of 2·1 was only slightly higher than that for a mixture of phospholipids.

(4) *Direct extraction with chloroform-methanol*

Results from extractions of sera from two salted butters (samples B and C) are presented in Table 2. The extracts after washing were found to contain no inorganic phosphate although the P contents were slightly higher than for the ether-light petroleum extracts. The contents of lecithin and sphingomyelin P were reasonably close to those of the ether-light petroleum extracts from the same sample. Although the N:P ratios of the chloroform-methanol extracts were higher than for phospholipids alone, the cephalin P result for sample B was very close to that for the extract from the colloidal iron precipitate and to that for the ether-light petroleum extract; and for sample C the value for cephalin P was fairly close to that for the ether-light petroleum extract. It appears, therefore, that washing the chloroform-methanol extract of the serum eliminates P compounds other than the phospholipids, thus confirming the work of previous investigators on brain and other tissue (22, 23, 24).

## DISCUSSION

The results indicate that the amount of P extracted from butter serum by ether-light petroleum mixture was slightly greater than that extracted by benzene-ethanol from the colloidal iron precipitate; and it was slightly less than that extracted by chloroform-methanol from the serum itself, or by ethanol-ether, benzene-ethanol and benzene-isopropanol from the TCA precipitate. Judging by the N:P ratio, no P compounds other than the phospholipids were extracted by the ether-light petroleum mixture from butter serum nor by benzene-ethanol from the colloidal iron precipitate. The N:P ratios of all other extracts were higher, indicating that other P compounds might be present. The slightly higher results obtained for lecithin P and for sphingomyelin P (calculated from their choline contents after hydrolysis) in some of the extracts compared with the ether-light petroleum extract, suggest that part, at least, of the increase in total P content was due to more efficient extraction of phospholipids.

In assessing any routine method of analysis, factors such as time consumed and manipulative skill required must be taken into consideration. Thus the possibility of a small error in the results from the ether-light petroleum extraction is of little consequence in comparison with the simple nature of the modified Röse-Gottlieb procedure. For routine analysis of butter samples, therefore, this method has been adopted.

## SUMMARY

A method has been described for the routine estimation of total phospholipids and of lecithin, cephalin and sphingomyelin in butter. Butter serum was treated with ether and light petroleum in a modified Röse-Gottlieb procedure to extract the phospholipids which then were estimated in the extract.

Chloroform-methanol extracted from the butter serum slightly more phosphorus than did the ether-light petroleum mixture. Ethanol-ether, benzene-ethanol and benzene-isopropanol, when used to extract a protein-phospholipid precipitate formed by the addition of trichloroacetic acid to butter serum, also removed slightly more phosphorus than did the ether-light petroleum mixture. The higher phosphorus contents of these extracts was due partly to a slightly more efficient extraction of phospholipids and partly to extraction of non-phospholipid phosphorus compounds.

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# 708. PHOSPHOLIPIDS IN NEW ZEALAND DAIRY PRODUCTS

## II. SEASONAL VARIATIONS IN THE PHOSPHOLIPID CONTENT OF BUTTER AND OF MILK AND CREAM

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(With 5 Figures)

The phospholipid content of butter is related to the phospholipid content of the cream from which it is derived. Any seasonal variation in the phospholipid content of milk should be reflected in some corresponding seasonal changes in the phospholipid content of butter.

Variations in the phospholipid content of milk with stage of lactation or with season already have been reported (1,2,3,4). There is general agreement that the phospholipid content is highest during the first few days of lactation. Baliga & Basu (4) found that in the milk of Sindhi cows the phospholipid content was normal within 7 days after parturition. It fell to a minimum during the second and third months of lactation and then rose gradually until at the end of lactation it was approximately the same as in colostrum milk. The same authors found little difference between average results for winter, summer and 'rains' seasons (4).

Apart from variations caused by seasonal changes in phospholipid content of milk the phospholipid content of butter may fluctuate with variations in the manufacturing conditions, causing differences in the proportion of phospholipid passing into the buttermilk during churning. It has been shown, for example, that the state of dispersion of the fat in cream is affected by conditions of pasteurization (5), and this would affect the phospholipid content of butter.

### COLLECTION OF SAMPLES

#### (1) *Butter for survey of seasonal changes*

Butter samples (1 lb. pats) were obtained twice monthly during the periods shown in Table 1 from two dairy factories in the Manawatu district of the North Island. The samples were received at the Institute within a few hours of manufacture and were stored at $-10°$ C. until required for analysis. (Storage at this temperature has no effect on the phospholipid content of butter, see Part III of this series (6).)

Table 1. *Butter-sampling periods at factories A and B, and analyses performed on the samples*

| Factory | Sampling period | Phospholipids estimated |
|---|---|---|
| A | November 1953–February 1957 | Total phospholipids |
| A | February 1955–July 1956 | Lecithin, cephalin and sphingomyelin |
| B | February 1955–February 1957 | Total phospholipids |
| B | February 1955–July 1956 | Lecithin, cephalin and sphingomyelin |

(2) *Milk, skim-milk and cream, pasteurized cream and buttermilk, for investigation of cause of seasonal changes*

These were obtained once or twice monthly over the period February 1956 to February 1957. At factory A samples of mixed whole milk, and of skim-milk and cream from the separation of the whole milk at 80–110° F. (pre-heating temperatures varied with the season of the year), were obtained. On the following day samples of pasteurized cream and buttermilk were taken from a churning of a mixture of this factory-separated cream and farm-separated cream pasteurized in a Vacreator* vacuum pasteurizer and held overnight.

At factory B samples of cream and buttermilk were obtained from one churning of mixed farm- and factory-separated cream on the same day as the corresponding samples from factory A.

At both factories the samples of cream and buttermilk were taken from the same churnings as those from which the butter samples were taken for the seasonal survey (see (1), above).

## EXPERIMENTAL

### *Extraction and estimation of phospholipids*

(1) *Butter*

Phospholipids were extracted from the butter serum and the total and individual phospholipid contents of the extract were estimated as described in Part I (7).

(2) *Milk, skim-milk, cream and buttermilk*

The phospholipids were extracted with ether and petrol ether according to the accepted procedure for the Mojonnier modification of the Röse-Gottlieb extraction of fat in milk and its products. This method, which has already been used by many workers in investigations on phospholipids in dairy products, was found to give reliable results, and of all the methods studied was the most easily adapted for routine analysis (7).

For milk, skim-milk and buttermilk 10 ml. (10·2 g.) were taken, and for cream 1 g. of sample was taken and diluted with 9 ml. of water. The mixed ethers from three extractions were combined and made up to a final volume of 100 ml. A small portion (1 ml. for buttermilk, 2 ml. for cream and milk and 5 ml. for skim-milk) was withdrawn for estimation of phospholipids, and the remainder was used for estimation of fat.

For estimation of total phospholipids the required volume of extract was freed of solvents in a Pyrex test tube and the residue was digested with 10 N sulphuric acid and 30% hydrogen peroxide, as already described for butter serum extracts (7). Where required to complete the digestion of milk and cream extracts, further quantities of hydrogen peroxide were added. The phosphorus content of the digest was estimated by the procedure used for the digest from butter serum extract (7).

The contents of lecithin, cephalin and sphingomyelin in the extracts were estimated by the method used in Part I for butter serum extracts (7).

* 'Vacreator' is the registered trade mark of the Murray vacuum pasteurizer manufactured by Murray Deodorizers Ltd., Auckland, New Zealand.

*Calculation of results*

Analytical results for percentage of extracted total phosphorus were converted to percentage of total phospholipids in each product by use of the factor 25·5 (7), and analytical results for percentage of extracted lecithin, cephalin and sphingomyelin phosphorus were converted to percentage of individual phospholipids by use of the factors 25·5, 24·4 and 26·8 respectively (7).

The proportions of cream and skim-milk produced in the separation of whole milk were calculated by the formula:

$$\text{quantity of skim-milk} = (C - M)/(C - S) \times 100, \quad (8a),$$

$$\text{quantity of cream} = 100 - \text{weight of skim-milk},$$

where $C$ = fat test of cream, $M$ = fat test of milk, and $S$ = fat test of skim-milk.

The proportions of buttermilk and butter produced in the churning of the cream were calculated from the following formulae:

$$\text{quantity of butter} = 1{\cdot}2F \quad (8b),$$

$$\text{quantity of buttermilk} = 100/(100 - f) \times (100 - 1{\cdot}12F + 0{\cdot}0012fF), \quad (8c),$$

where $F$ = fat content of cream, $f$ = fat content of buttermilk.

## RESULTS

### (1) *Butter*

(*a*) *Unweighted average total and individual phospholipid contents.* The unweighted average phospholipid content of all butters sampled over the whole period of the investigation, and the average individual phospholipid content of butters sampled during the period February 1955 to July 1956, along with the minimum and maximum results for each group, are given in Table 2.

Table 2. *Unweighted average total and individual phospholipid contents of butter*

| Period of sampling | November 1953–February 1957 | | | February 1955–July 1956 | | |
|---|---|---|---|---|---|---|
| No. of samples ... ... | 130 | | | 72 | | |
| | Average | Minimum | Maximum | Average | Minimum | Maximum |
| Total phospholipids (%) | 0·133 | 0·099 | 0·181 | 0·136 | 0·099 | 0·181 |
| Lecithin (%) | — | — | — | 0·038 | 0·025 | 0·050 |
| Cephalin (%) | — | — | — | 0·051 | 0·032 | 0·072 |
| Sphingomyelin (%) | — | — | — | 0·047 | 0·035 | 0·062 |

The averages for both factories, weighted to allow for variation in monthly butter production, for the period August 1955 to July 1956 were: total phospholipids, 0·139%; lecithin, 0·041%; cephalin, 0·051%; sphingomyelin, 0·047%.

These figures may be taken as representing the annual average phospholipid content of the butters produced at factories A and B.

(*b*) *Seasonal variations.* The unweighted monthly average total phospholipid contents of the butters from each factory over the whole period of the investigation are shown in Fig. 1. There was a marked seasonal variation, with a maximum in late summer and autumn (February–March) and a minimum in the winter (June–August). For butters from factory B the rise to a maximum and the fall to a minimum in each year were steady,

but for butters from factory A the uniform rise from July onwards was arrested each year by a temporary fall in November or December.

The curves for unweighted monthly average lecithin, cephalin and sphingomyelin contents of butter at these factories over the period February 1955 to July 1956, shown in Fig. 2, follow much the same trend for each phospholipid as the curves for total phospholipid content.

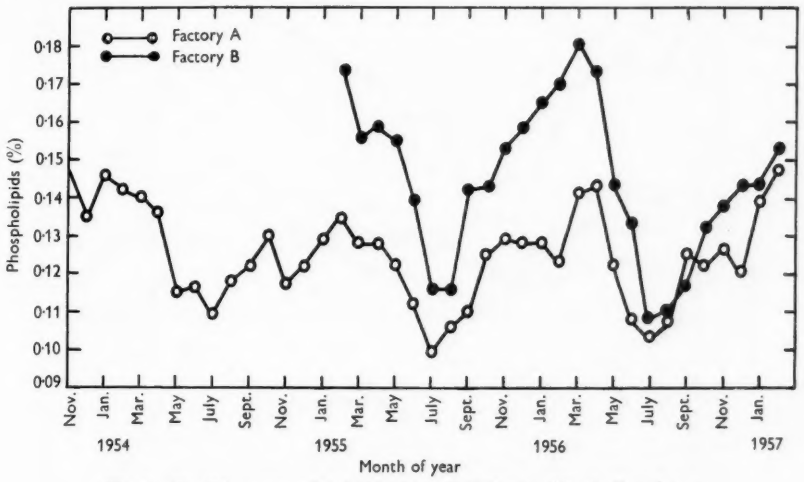


Fig. 1. Monthly average total phospholipid contents of the butters from factories A and B. ○—○, factory A; ●—●, factory B.

(*c*) *Proportions of lecithin, cephalin and sphingomyelin in total phospholipids of butter.* The results are shown in Table 3. There was a considerable range of variation in the proportions of lecithin, cephalin and sphingomyelin in the total phospholipids of the butters from both factories. Nevertheless, the average proportion of each phospholipid at factory A was very similar to the corresponding figures for factory B. Hence the average proportions of lecithin, cephalin and sphingomyelin in the total phospholipids of all butters analysed were 28, 38 and 34% respectively.

Table 3. *Proportions of lecithin, cephalin and sphingomyelin in the total phospholipids of butter over the period February* 1955–*July* 1956

| | Proportion of total phospholipids (%) | | | | | |
|---|---|---|---|---|---|---|
| | Factory A | | | Factory B | | |
| Phospholipid | Average | Minimum | Maximum | Average | Minimum | Maximum |
| Lecithin | 28·9 | 23·2 | 32·8 | 27·8 | 23·3 | 31·9 |
| Cephalin | 37·3 | 32·3 | 41·8 | 37·6 | 34·4 | 39·7 |
| Sphingomyelin | 33·8 | 31·2 | 37·7 | 34·6 | 32·7 | 38·4 |

(2) *Milk, skim-milk and cream*

(*a*) *Average total phospholipid contents* (see Table 4). The unweighted average total phospholipid contents of the milk, skim-milk and cream were 0·038, 0·018 and 0·181%, and the average proportions of phospholipid in the fat in each product were 0·80, 22·5

and 0·44%, respectively. The proportion of phospholipid passing from the whole milk to the skim-milk varied from 40·6 to 48·0%, average 44·2%.

(*b*) *Seasonal variations.* Results for the phospholipid contents of milk and skim-milk and for the percentages of phospholipids in the fats of the milk and cream at each sampling

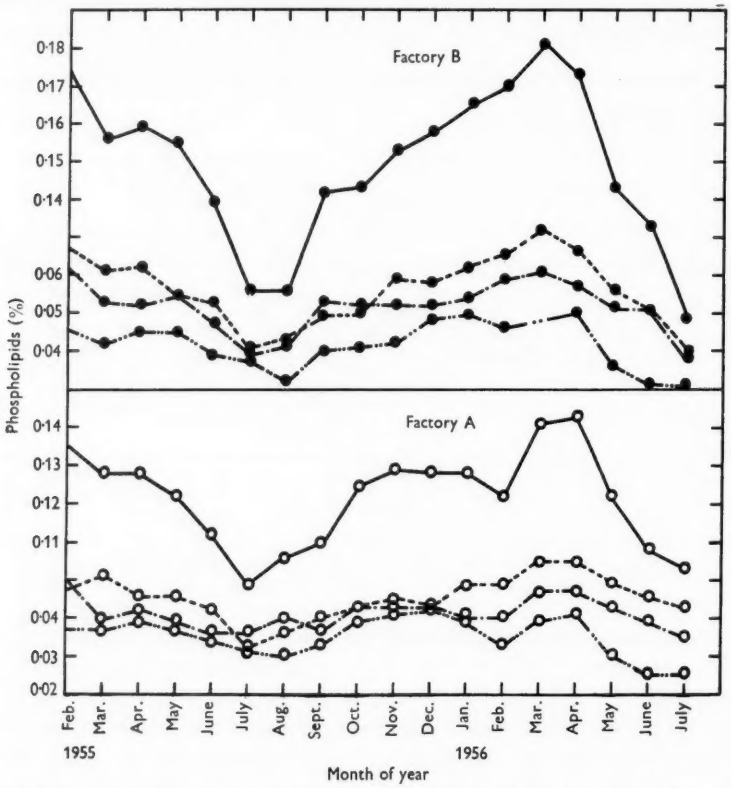


Fig. 2. Monthly average lecithin, cephalin, sphingomyelin and total phospholipid contents of the butters from factories A and B. Factory A: ○—○, total phospholipids; ○—·—○, lecithin; ○– – –○, cephalin; ○—·—○, sphingomyelin. Factory B: ●—●, total phospholipids; ●—·—●, lecithin; ●– – –● cephalin; ●—·—●, sphingomyelin.

Table 4. *Fat and total phospholipid contents of nineteen samples of milk, skim-milk and cream*

| | Milk | Skim-milk | Cream |
|---|---|---|---|
| Fat content (%) | | | |
| Average | 4·71 | 0·081 | 41·5 |
| Minimum | 4·43 | 0·065 | 36·7 |
| Maximum | 5·59 | 0·120 | 48·6 |
| Total phospholipid content (%) | | | |
| Average | 0·038 | 0·018 | 0·181 |
| Minimum | 0·031 | 0·014 | 0·149 |
| Maximum | 0·050 | 0·023 | 0·247 |
| Total phospholipids in fat (%) | | | |
| Average | 0·80 | 22·5 | 0·44 |
| Minimum | 0·70 | 13·3 | 0·38 |
| Maximum | 0·98 | 28·6 | 0·51 |

date are plotted in Fig. 3. The phospholipid contents of milk and skim-milk show very similar trends—the figures were at a maximum in the late autumn (April and May) and at a minimum in the spring (September–October). After a slight rise in October there was little change until February.

The proportion of phospholipids in the fat of milk and cream was at a maximum in April (see Fig. 3). In milk the proportion decreased from June to August and then varied only slightly until February. In cream the proportion showed no great variation from May onwards.

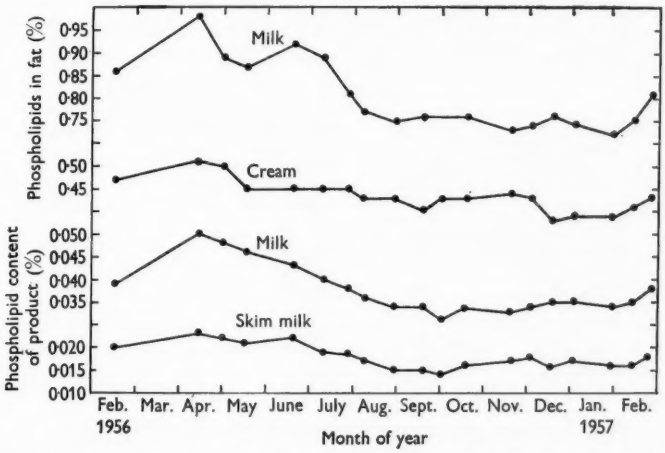


Fig. 3. The phospholipid contents of milk and skim-milk and the proportions of phospholipids in the fats of milk and cream for each sampling date.

(*c*) *Results for gravity separation of milk.* A sample of whole milk from the bulk supply at factory A was allowed to stand at 10° C. for 48 hr. The fat content of the skim-milk was 0·244% and the phospholipid content was 0·017%. Results for centrifugal separation of the milk at the factory were: fat, 0·093%; phospholipids, 0·016%. Although the fat and the phospholipid contents of the gravity-separated cream were lower than those of the centrifugally-separated cream the proportion of phospholipids (0·44%) was the same in the fats of both creams.

(*d*) *Results for separator slime.* Samples of slime from a farm separator and from a factory separator contained 0·78 and 0·69% of phospholipids respectively. The weight of slime represented not more than 0·05% of the weight of milk separated. The phospholipid content of skim-milk would therefore be affected only slightly by even quite large variations in phospholipid content of the slime.

### (3) *Pasteurized cream and buttermilk*

(*a*) *Average total phospholipid contents* (see Table 5). The average phospholipid contents of the creams and buttermilks sampled at factory A were 0·155 and 0·164%, and at factory B were 0·154 and 0·147%. The average proportions of phospholipids in the fats of the creams were 0·41% at factory A and 0·40% at factory B, and in the fats of the buttermilks 18·3 and 15·8% respectively. At factory A the average proportion of

phospholipids lost from the cream to the buttermilk was 61·8% (57·0–67·3%) and at factory B 57·1% (51·2–62·0%).

(*b*) *Seasonal variations.* Seasonal variations in the phospholipid contents of cream, buttermilk and butter sampled from the same churnings are shown in Fig. 4 for both factories. The lines show a similar trend for the three products, with maximum values in the autumn, a fairly rapid decline to minimum values in the winter (June–July), and a gradual rise from then to the following autumn. The proportions of phospholipids in the fat in cream and butter (see Fig. 5) also follow the same trend.

Table 5. *Fat and total phospholipid contents of twenty-one samples of cream and buttermilk from each of the factories A and B*

| | Factory A | | Factory B | |
|---|---|---|---|---|
| | Cream | Buttermilk | Cream | Buttermilk |
| Fat content (%) | | | | |
| Average | 37·5 | 0·92 | 37·5 | 0·94 |
| Minimum | 33·5 | 0·74 | 32·8 | 0·69 |
| Maximum | 40·8 | 1·21 | 40·0 | 1·15 |
| Total phospholipid content (%) | | | | |
| Average | 0·155 | 0·164 | 0·154 | 0·147 |
| Minimum | 0·122 | 0·126 | 0·111 | 0·103 |
| Maximum | 0·183 | 0·191 | 0·189 | 0·182 |
| Total phospholipids in fat (%) | | | | |
| Average | 0·41 | 18·1 | 0·40 | 15·8 |
| Minimum | 0·36 | 13·4 | 0·35 | 12·9 |
| Maximum | 0·48 | 24·5 | 0·49 | 23·0 |

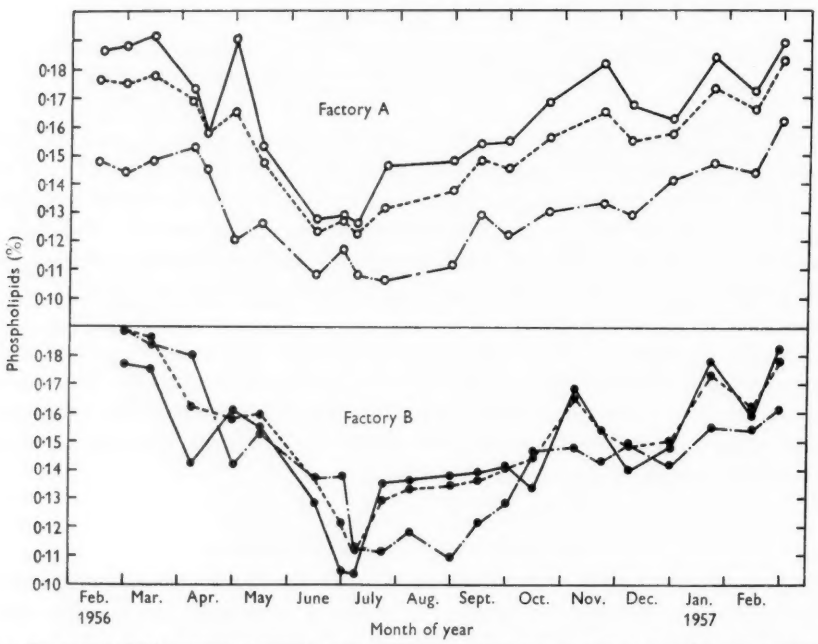


Fig. 4. The phospholipid contents of creams, buttermilks and butters from factories A and B for each sampling date. Factory A: ○---○, cream; ○—○, buttermilk; ○—·—○, butter. Factory B: ●---●, cream; ●—●, buttermilk; ●—·—●, butter.

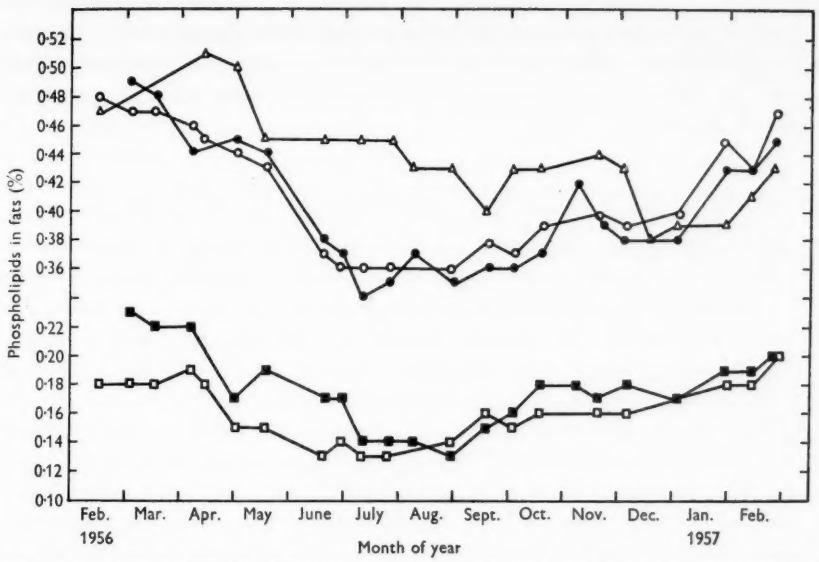


Fig. 5. The proportions of phospholipids in the fats of creams and butters from factories A and B and in the fats of factory-separated raw creams from factory A for each sampling date. △—△, cream, factory-separated; ○—○, cream, factory A; ●—●, cream, factory B; □—□, butter, factory A; ■—■ butter, factory B.

### (4) *Comparison of proportions of lecithin, cephalin and sphingomyelin in the total phospholipids of skim-milk, buttermilk and butter*

The average individual phospholipid contents and the average percentage proportions of each phospholipid in the total phospholipids of samples of skim-milk and butter taken from factory A during three sampling periods, and for samples of buttermilk and butter taken from a churning on three different sampling dates, are shown in Table 6. Samples of milk and cream also were analysed for individual phospholipids but, because of the relatively large amounts of fat present in the extracts, hydrolysis of lecithin and cephalin with potassium hydroxide was incomplete, and the results were unreliable.

Table 6. *Average total and individual phospholipids in skim-milk and butter, and in buttermilk and butter*

| | Skim-milk | Butter | Buttermilk | Butter |
|---|---|---|---|---|
| Phospholipids in product (%) | | | | |
| Total phospholipid | 0·019 | 0·116 | 0·158 | 0·142 |
| Lecithin | 0·006 | 0·034 | 0·043 | 0·040 |
| Cephalin | 0·007 | 0·047 | 0·068 | 0·058 |
| Sphingomyelin | 0·006 | 0·035 | 0·047 | 0·044 |
| Proportion of individual phospholipids in total phospholipids (%) | | | | |
| Lecithin | 31·6 | 29·3 | 27·2 | 28·2 |
| Cephalin | 36·8 | 40·5 | 43·0 | 40·8 |
| Sphingomyelin | 31·6 | 30·2 | 29·7 | 31·0 |

Average results for the proportion of each phospholipid in the total phospholipids of skim-milk and butter, and also of buttermilk and butter, agreed fairly well. Hence it may be assumed that after separation of milk and churning of the cream the proportion of each

phospholipid in the total phospholipids of the products will be fairly close to that in the milk itself. Thus results reported earlier in the paper (see Results, 1 (*d*)) for average percentage proportions of lecithin, cephalin and sphingomyelin in the total phospholipids of butter will indicate fairly accurately the average values for milk, skim-milk, cream and buttermilk also.

## DISCUSSION

### (1) *Comparison of phospholipid contents with those reported by other authors*

(*a*) *Butter.* The average (unweighted) total phospholipid content of butter was of the same order as the average values reported by Crane & Horrall (9), Horrall (3), Heinemann (10) and Smith (11), all of whom extracted the phospholipids from butter by the Röse-Gottlieb procedure. The average contents of total phospholipids and of lecithin, cephalin and sphingomyelin were similar also to those reported by Baliga & Basu (12), who extracted the phospholipids from butter with boiling petrol ether.

(*b*) *Milk.* The average total phospholipid content of milk (0·038 %) was slightly higher than the values reported by Horrall (0·034 % (3)) and Crane & Horrall (0·032 % (9)), who used the Röse-Gottlieb method for extracting the phospholipids; and higher than that reported by Baliga & Basu (0·031 % (4)) who extracted the milk with alcohol-ether, evaporated off the solvent and then extracted the phospholipids from the residue with boiling petrol ether.

(*c*) *Skim-milk and cream.* The average phospholipid contents of skim-milk and cream agreed fairly closely with the results reported by Horrall (3) and by Heinemann (10), both of whom estimated the phospholipids in the Röse-Gottlieb extracts.

(*d*) *Buttermilk.* The average result (0·156 %) and the range of results (0·111–0·189 %) for total phospholipid contents of all buttermilks sampled at factories A and B agreed fairly closely with the figures (0·149 % (0·103–0·198 %)) obtained by Bird, Breazeale & Sands (13) using the Röse-Gottlieb extraction method for phospholipids in seventy-four buttermilks from both sweet and sour cream churnings in United States. They found no evidence of seasonal variation in phospholipid content of buttermilk.

### (2) *Seasonal variations*

(*a*) *Milk.* It has been shown that there were seasonal variations in the phospholipid content of milk, and that phospholipid contents of skim-milk, cream, buttermilk and butter all followed the same trend as for milk. Since the stage of lactation of the cow influences the phospholipid content of the milk (4), these seasonal variations probably were due to the seasonal nature of dairying for buttermaking in New Zealand. In early lactation the fat globules would be much larger than in late lactation (8 *d*), and the amount of phospholipid required to seal off all the globules would be considerably less (14). Thus in September when the milk was drawn almost entirely from cows in the first or second month of lactation the phospholipid content was low, but in March, April and May when the cows were in late lactation it was higher than at any other time of the year. The decline in values in June, July and August probably was due to the increasing quantity of milk from cows in early lactation and to the decreasing quantity from cows in late lactation. It is surprising, however, that early post-colostral milk, which would reach its maximum proportion in the mixed milk in late July or in August (15), apparently had little effect on the phospholipid content.

Other workers have found that as lactation progresses from the third month onwards there is a decrease in the size of the fat globules (8*d*), and that the phospholipid content of the milk increases (4). In the present investigation, however, there was little change in the phospholipid content of milk, or in the proportion of phospholipids in the fat of the milk, from September to February (see Fig. 3).

(*b*) *Skim-milk and cream.* Although the phospholipid contents of the skim-milks showed the same seasonal trend as those of the whole milk, the fat contents varied irregularly from sample to sample. Also, in spite of a much higher fat content, the phospholipid content of gravity-separated skim-milk was only slightly higher than that of factory-separated skim-milk. Thus it appears that increases in fat content of skim-milk above a certain minimum were due to inclusion of relatively large fat globules which increased the phospholipid content only slightly.

It seems probable, therefore, that seasonal trends in phospholipid content of the skim-milk were due to variations in the phospholipid content of the small fat globules which resisted centrifugal separation. It was calculated by Sommer (14) that in globules of this size the mono-layer of phospholipids on the fat globule surface would represent an appreciable proportion of the total weight of the globule. Nevertheless, in many of the skim milks analysed during the present investigation the proportion of phospholipids in the fat was far too high to be explained satisfactorily by concentration of the phospholipids only at the surface of the fat globules. Possibly, as Lea suggests (16), the smaller fat globules are really lipoprotein particles or 'milk microsomes' (as described by Morton (17)) in which the proportion of phospholipids is very high.

In cream the phospholipids are mainly concentrated at the fat globule surface and the amount of phospholipid per unit of fat will vary with the average size of the fat globules. Thus the seasonal variations in phospholipid content of the cream separated at factory A probably were correlated with stage of lactation of the herds from which the milk was drawn.

(*c*) *Pasteurized cream, buttermilk and butter.* Low phospholipid contents of cream and buttermilk during the winter months were due partly to a reduction in the fat content of the cream because of greater dilution with water both at the farm and at the factory. Butter, however, is relatively constant in fat content, and its phospholipid content was found in trials at this Institute not to be affected by dilution of the cream with 4 and 8% of water before churning. Since the proportions of phospholipids lost from the cream to the buttermilk was fairly constant at factory A and at factory B it is apparent that normal variations in butter-making conditions had little effect on the phospholipid contents of the buttermilk or the butter. Thus the phospholipid contents of the buttermilks followed the same trend as those of the creams (see Fig. 4), and the phospholipid contents of the butters were influenced mainly by the seasonal variations in the amount of phospholipid per unit of fat in the cream (see Fig. 5).

### (3) *Comparison of phospholipid contents of factory-separated and farm-separated creams*

The proportion of phospholipids in the fat of factory-separated raw cream from factory A was for the greater part of the year higher than that of the mixed farm- and factory-separated pasteurized cream* sampled from churnings at factories A and B

* The proportion of factory-separated cream in the mixed creams of any one churning at either factory A or factory B probably was never greater than 50% and often was considerably less.

(see Fig. 5). Since pasteurization had no effect on the phospholipid content of the factory-separated cream and since the whole milk used for farm- and factory-separation was drawn from the same district, the difference in phospholipid contents of the creams can be explained only by assuming that there was a greater loss of phospholipids from the whole milk to farm skim-milk than to factory skim-milk. Samples of factory skim-milk showed an average fat content of 0·081% over 12 months but samples of skim-milk collected by tanker from farms elsewhere in the North Island showed an increase in fat content (estimated by the Röse-Gottlieb method) from 0·08% in December to 0·10% in March and to 0·12% or even higher, in May. It has been shown earlier in this paper, however, that increased fat losses in skim-milk had only a minor effect on the phospholipid contents of the skim-milk or the cream. Thus the greater loss of phospholipids from whole milk to farm skim-milk probably was due to a greater disruption of fat globules during separation. This could have been caused by the type of separator used, or by separating the milk at too low a temperature, especially during the winter and early spring when milk is preheated to temperatures of 95–110° F. at the factory.

### (4) *Comparison of total phospholipid contents of buttermilks and butters from factories A and B*

The average proportion of phospholipids lost from the cream to the buttermilk was greater at factory A than at factory B. Since the proportion of phospholipids in the fats of creams from both factories agreed fairly closely on most sampling dates (see Fig. 5), the phospholipid contents of factory B butters were, almost without exception, higher than those of factory A (see Figs. 1, 2 and 5).

Average results for contents of total fat, phospholipids and 'triglyceride' fat (total fat % − phospholipids %) in the buttermilks from factories A and B and for percentage losses for each of these 'fats' from the creams to the buttermilks are shown in Table 7. In spite of the greater loss of phospholipids from the creams to the buttermilk at factory A the losses of total fat and 'triglyceride' fat were slightly less than at factory B.

Table 7. *Average contents of total fat, total phospholipids and 'triglyceride' fat in buttermilks from factories A and B*

| | Factory A | Factory B |
|---|---|---|
| Total fat | | |
| In buttermilk (%) | 0·921 | 0·944 |
| As % of fat in cream | 1·425 | 1·458 |
| Total phospholipids | | |
| In buttermilk (%) | 0·164 | 0·147 |
| As % of fat in cream | 0·254 | 0·227 |
| 'Triglyceride' fat | | |
| In buttermilk (%) | 0·757 | 0·797 |
| As % of fat in cream | 1·171 | 1·231 |

Factors which could have influenced the loss of phospholipids from the cream to the buttermilk were (*a*) intensity of treatment of the cream in the Vacreator during pasteurization, and (*b*) variations in buttermaking technique.

(*a*) Almost without exception during the sampling period the intensity of treatment was greater at factory A than at factory B. Thus a greater disruption of fat globules in factory A cream could have led to a greater loss of phospholipids from the globule surface

to the cream serum. Unfortunately there is no experimental evidence to support this theory.

(*b*) The quantity of phospholipids transferred from the fat globule to the buttermilk during churning could possibly have been influenced by differences in buttermaking conditions. It was found, however, that variations in churning temperature, in size of butter granules, in washing treatment of the butter and in the size of the churn had little effect on the phospholipid loss and would not explain the differences in values between the two factories.

SUMMARY

Samples of milk, skim-milk and raw cream from one factory and of pasteurized cream, buttermilk and butter from two factories were taken twice monthly for varying periods for estimation of total phospholipids and (in butter only) of lecithin, cephalin and sphingomyelin.

Seasonal variations in the phospholipid contents of milk, skim-milk and raw cream over 13 months probably were due to the greater proportion of small fat globules in autumn (late lactation) milk compared with spring (early lactation) milk.

There were regularly recurring seasonal variations in total phospholipid content of the butters over 3 years. Maximum results were found during autumn and minimum results in winter. Lecithin, cephalin and sphingomyelin contents followed the same trends as the total phospholipid contents.

Seasonal variations in phospholipid contents of buttermilks and butters were due mainly to variations in the amount of phospholipid per unit of fat in the cream. Differences in butter-making technique had little effect on the proportion of phospholipids from the cream retained in the butter.

Average results for total phospholipid content of the products examined were: milk, 0·038% (0·031–0·050%); skim-milk, 0·018% (0·014–0·023%); buttermilk, 0·156% (0·103–0·191%); butter, 0·133% (0·099–0·181%). The average result for percentage of total phospholipid in the fat of the raw creams examined was 0·44% (0·38–0·51%); and in the fat of the pasteurized creams 0·41% (0·35–0·49%). Average weighted results for total phospholipid and for lecithin, cephalin and sphingomyelin contents of butter were 0·139, 0·041, 0·051 and 0·047% respectively.

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# 709. THE CALORIFIC VALUE OF MILK OF DIFFERENT SPECIES

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The gross calorific value of milk is important in studies of both human and animal nutrition. It is necessary in calculations on the feed requirements of milking animals for the maintenance of lactation and in studies on breeding of dairy cows for increased milk production. Direct determination of calorific value of milks has not been done extensively, probably on account of the tedious nature of the analyses required and the perishable nature of milk samples.

Various combustion factors per gram of constituent have been reported for cow's milk. The standard values directly determined, as given by Abderhalden (1) in 1908 are: fat 9·23, protein 5·71, and lactose 3·95 Cal./g. These values appear to be the result of work done some years earlier. In 1926 Andersen (2) reported the values 4132, 2658 and 1792 Cal./lb. of milk fat, milk protein and lactose respectively. These values correspond to fat 9·11, protein 5·86 and lactose 3·95 Cal./g., and agree closely with those reported earlier. Overman & Sanmann (3) compared the directly determined energy content and chemical composition of 212 samples and obtained relationships between calorific content and the various milk components. Later Overman & Gaines (4) analysed the same data and also a more extensive series. They reported that the values for the calorific content of milk fat, milk protein and lactose fluctuated to some extent according to whether the ash and water contents of the milk were included. They listed the following values based on fat, protein and lactose only: fat $9{\cdot}253 \pm 0{\cdot}065$, protein $5{\cdot}853 \pm 0{\cdot}127$ and lactose $3{\cdot}69 \pm 0{\cdot}059$ Cal./g. Overman & Sanmann concluded by suggesting that the true heats of combustion per gram of fat, protein and lactose for the milk of the dairy cow probably lie between the values given by Abderhalden and those found by Andersen. The present paper follows Andersen in using the factors 9·11, 5·86 and 3·95 Cal./g. for fat, protein and lactose contents in all computed energy values.

Very few corresponding values for the milk of other species have been reported and in many nutrition studies it is customary to use the appropriate cow milk conversion factors despite probable species differences.

Petersen & Turner (5) reported the composition and calorific value as directly determined of eighteen samples of goat's milk. Although the mean energy value (from calorimetry) was within 1% of the average calculated value from the fat, protein and lactose contents of the eighteen samples, the series included one result for which the estimated energy value was 20% higher and two results for which the estimated energy values were 16 and 28% lower than the corresponding energy values as directly determined. For the remaining samples there was good agreement between the two estimates. No data appear to exist relating determinations of calorific value and chemical composition of ewe's milk.

The only observation on directly determined energy content of sow's milk of known composition is that of Vinogradsky (6). Estimating lactose contents by difference, in

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order to calculate energy value from chemical composition, gave agreement to within 6% for eight of the ten sets of analyses listed. In six of these eight sets, the directly determined energy value was greater than the calorific value calculated from the milk composition. For the two sets in which values did not agree within 6% the calorific values determined directly were 14 and 16% higher than those calculated from milk composition.

The gross calorific values of three samples of human milk listed by Shukers *et al.* (7) are similar to the values calculated from the chemical composition. Hytten (8), in 1954, reported that the absolute calorific equivalents of human milk are not known and he used the factors 9·0, 4·0 and 3·75 Cal./g. for fat, protein and lactose respectively in his estimates of calorific value.

In view of these differences, an investigation was undertaken in association with other milk composition studies, of the gross energy value of milks as determined by bomb calorimeter and the corresponding energy values as computed from milk composition. A likely source of error was considered to be the inclusion in the protein value (N × 6·38) of the non-protein nitrogen content which had been found earlier to vary appreciably between species and slightly during the course of lactation. Preliminary work gave the non-protein nitrogen as 5–7% of the total nitrogen in the milk of the cow, goat and ewe, about 14% for milk of the sow and up to 20% in human milk. Samples were therefore analysed not only for fat, total solids, lactose and ash contents but also for both total and non-protein nitrogen, and the calorific value was directly determined.

## EXPERIMENTAL

*Dairy cow.* Milk samples were obtained from the experimental herds of this Research Station. All samples were from individual animals and were night–morning composite samples combined in the proportion of their respective yields and analysed when fresh. Samples were taken throughout the normal lactation periods of the cows.

*Sow.* Samples were obtained from Berkshire sows by machine milking, using Pitocin injection to stimulate let-down (9).

*Ewe.* Samples were taken by hand-milking from one half of the udder while the lamb was allowed to suckle the other. This procedure had been found satisfactory in other studies on ewe milk composition. The sheep were all 5-year-old New Zealand Romney ewes.

*Goat.* Samples representative of the complete hand-milking of a goat were obtained once a week from the second to the sixth week of lactation.

*Human.* Samples from the complete emptying of one breast were obtained 2, 5 and 12 days and 4, 7 and 10 weeks *post partum.*

Determinations of the percentages of fat, total solids, total nitrogen, non-protein nitrogen, lactose, ash and gross energy value were obtained for all the samples studied. Fat content was determined by the Gerber method and total solids by drying at 98–100° C. Solids-not-fat were obtained by difference. Total nitrogen and non-protein nitrogen were determined by methods similar to those described by Rowland (10). The standard semi-micro Kjeldahl procedure was used for all nitrogen estimations. Lactose was determined by a modification of the volumetric copper reduction method of Lane & Eynon (11), the milk being clarified by 10% lead acetate and saturated sodium fluoride solutions. Ash was determined by drying 10 g. samples in an oven at 98–100° C. overnight and then incinerating in a muffle furnace below 550° C. to constant weight.

A Bomb Calorimeter (Baird & Tatlock Ltd.) was used for the determinations of gross energy values. The water equivalent of the calorimeter was accurately determined by combustions of weighed amounts of pure fused benzoic acid. Samples of milk were accurately weighed into Vitreosil crucibles (squat form, capacity about 15 ml.) and dried under reduced pressure at 70° C. (Earlier trials showed that a sufficiently large sample of milk could not be dried adequately in the tall form of silica crucible supplied with the calorimeter and that prolonged drying in either the tall or squat form of crucible in air at 98–100° C. led to overheating of some of the milk constituents and a slightly lower calorific value.) Where possible, the quantity of milk used was chosen to give a temperature increment on incineration of about 2° C., that is, a heat change corresponding to 4000 to 5000 cal. Thus, samples were about 5 g. for the sow and ewe and 7 g. for the cow and goat.

The dried samples were stored in a desiccator until calorimetric measurement could be made. A platinum fuse wire (S.W.G. no. 44) was used. Combustions were made in oxygen at a pressure of 30 atmospheres. Temperature changes were read to 0·001° C. on a Beckman thermometer calibrated against the N.P.L. certificated standard. To correct the observed rise in temperature for the heat lost during the course of the experiment, the cooling correction calculated from the formula of Regnault & Pfaundler (12) was used. Since the maximum temperature was frequently not attained until the sixth or seventh minute from the time of firing, it was found advisable to continue readings for a further 5 min. in order to evaluate the rate of cooling during this final period. At least half an hour after the completion of readings, during which time the acid mist had settled, the bomb was opened and its contents analysed for total acidity and nitric acid content. (In practice, it was found a considerable advantage to have a second bomb, bomb stand and inner water container, and to alternate the use of these two outfits in order to allow adequate time for solution of the sulphuric and nitric acids formed during the combustion of the milk sample. The water equivalents of both bomb outfits were evaluated independently.) A necessary correction to be applied was the allowance for the small amount of unburnt carbon. This was obtained by oven drying the crucible after ignition, and determining the loss of weight following incineration in a muffle furnace. The observed weight difference in mg. was multiplied by 8·137 cal. This correction was generally of the order of 0·5%. Measurements where the amount of unburnt carbon exceeded 4 mg. were repeated. Other corrections for the heat liberated by the cotton fuse (approximately 12 cal.) and by the formation of nitric acid (up to 0·5%) as well as for the heat absorbed in the formation of sulphuric acid (up to 0·5%) were made as recommended in the handbook supplied with the bomb calorimeter (12). The sulphuric acid correction for sow's milk corresponded to a sulphur content of 0·08–0·09%, a value several times greater than was found for the other species studied. All chemical analyses were in duplicate, and replicate determinations of gross energy value by bomb calorimeter were always made. Calorimetric measurements for replicates generally agreed within 0·4%.

## RESULTS AND DISCUSSION

Table 1 shows the chemical composition and the calculated and experimentally determined calorific values for milks of the five mammalian species studied. Forty-five samples of cow's milk were analysed, the table listing in order results for samples with the lowest

Table 1. *Analyses of milk samples, with determined and computed calorific values*

| | | Chemical composition (g./100 g.) | | | | | Calorific values (per 100 g. milk) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Fat | Total solids | Total N | Non-protein N | Protein | Lactose | Ash | Determined | Formula I | Formula II | Formula III |
| Cow (extreme values) | | | | | | | | | | |
| 2·13 | 10·36 | 0·563 | 0·042 | 3·32 | 4·03 | 0·78 | 54·18 | 56·38 | 54·78 | 55·09 |
| 7·35 | 17·06 | 0·871 | 0·054 | 5·21 | 3·60 | 0·83 | 110·88 | 113·75 | 111·71 | 112·11 |
| 5·08 | 12·66 | 0·620 | 0·038 | 3·71 | 3·17 | 0·69 | 80·80 | 81·99 | 80·54 | 80·82 |
| 5·43 | 15·37 | 0·723 | 0·058 | 4·24 | 4·85 | 0·81 | 94·72 | 95·67 | 93·47 | 93·90 |
| 6·10 | 14·49 | 0·553 | 0·038 | 3·29 | 4·32 | 0·74 | 93·82 | 93·32 | 91·91 | 92·20 |
| 6·80 | 16·38 | 0·967 | 0·054 | 5·82 | 2·75 | 0·91 | 106·77 | 108·98 | 106·92 | 107·32 |
| Average for 45 samples | | | | | | | | | | |
| 5·05 | 13·86 | 0·675 | 0·045 | 4·02 | 3·89 | 0·78 | 84·58 | 86·88 | 85·16 | 85·48 |
| Sow | | | | | | | | | | |
| 5·05 | 16·57 | 0·886 | 0·119 | 4·89 | 4·86 | 0·97 | 92·83 | 98·34 | 93·86 | 94·74 |
| 5·30 | 17·78 | 1·100 | 0·165 | 6·03 | 4·60 | 1·09 | 101·44 | 107·59 | 101·79 | 103·01 |
| 5·40 | 17·30 | 0·900 | 0·127 | 4·93 | 5·18 | 0·99 | 100·10 | 103·29 | 98·54 | 99·48 |
| 5·80 | 17·79 | 1·047 | 0·169 | 5·60 | 4·67 | 1·07 | 104·09 | 110·44 | 104·10 | 105·35 |
| 5·88 | 18·36 | 0·884 | 0·116 | 4·90 | 5·74 | 1·13 | 108·12 | 109·30 | 104·95 | 105·81 |
| 5·92 | 17·40 | 0·891 | 0·113 | 4·97 | 4·95 | 0·92 | 104·17 | 106·81 | 102·61 | 103·44 |
| 6·11 | 15·32 | 0·824 | 0·121 | 4·48 | 3·29 | 0·65 | 95·19 | 99·48 | 94·91 | 95·81 |
| 6·22 | 17·85 | 0·812 | 0·124 | 4·39 | 5·41 | 1·06 | 106·92 | 108·40 | 103·76 | 104·68 |
| 6·48 | 18·65 | 0·837 | 0·105 | 4·67 | 5·81 | 0·89 | 102·20 | 113·29 | 109·35 | 110·13 |
| 6·96 | 19·00 | 1·105 | 0·165 | 6·00 | 4·39 | 1·00 | 117·29 | 122·07 | 115·91 | 117·13 |
| 7·15 | 18·58 | 1·045 | 0·191 | 5·45 | 4·48 | 0·90 | 117·19 | 121·92 | 114·77 | 116·18 |
| Average for 11 samples | | | | | | | | | | |
| 6·02 | 17·69 | 0·939 | 0·138 | 5·12 | 4·85 | 0·97 | 104·50 | 109·54 | 104·05 | 105·07 |
| Ewe | | | | | | | | | | |
| 10·19 | 21·48 | 1·170 | 0·075 | 6·99 | 2·91 | 0·87 | 146·36 | 148·08 | 145·29 | 145·84 |
| 10·74 | 22·16 | 1·111 | 0·067 | 6·66 | 3·44 | 0·89 | 149·79 | 152·98 | 150·46 | 150·95 |
| 11·88 | 23·57 | 1·149 | 0·069 | 6·89 | 3·40 | 0·94 | 164·64 | 164·63 | 162·03 | 162·54 |
| 12·20 | 23·36 | 1·244 | 0·075 | 7·46 | 2·43 | 0·99 | 158·83 | 167·27 | 164·46 | 165·01 |
| Average for 4 samples | | | | | | | | | | |
| 11·25 | 22·64 | 1·171 | 0·072 | 7·00 | 3·05 | 0·92 | 154·91 | 158·24 | 155·56 | 156·09 |
| Goat | | | | | | | | | | |
| 5·50 | 15·01 | 0·668 | 0·041 | 4·00 | 4·51 | 0·84 | 91·11 | 92·90 | 91·36 | 91·66 |
| 5·60 | 15·55 | 0·732 | 0·042 | 4·40 | 4·51 | 0·86 | 94·71 | 96·21 | 94·61 | 94·93 |
| 5·78 | 15·96 | 0·748 | 0·049 | 4·46 | 4·68 | 0·84 | 96·93 | 99·12 | 97·28 | 97·64 |
| 7·37 | 17·80 | 0·798 | 0·050 | 4·77 | 4·55 | 0·86 | 112·54 | 114·96 | 113·07 | 113·43 |
| 7·92 | 18·66 | 1·030 | 0·064 | 6·16 | 3·36 | 0·96 | 120·85 | 123·95 | 121·52 | 121·99 |
| Average for 5 samples | | | | | | | | | | |
| 6·43 | 16·60 | 0·795 | 0·049 | 4·76 | 4·32 | 0·87 | 103·23 | 105·43 | 103·57 | 103·93 |
| Human colostrum | | | | | | | | | | |
| 0·93 | 12·28 | 0·721 | — | — | 6·34 | 0·33 | 59·95 | 60·48 | — | — |
| 5·13 | 14·51 | 0·321 | 0·055 | 1·70 | 7·09 | 0·31 | 87·88 | 86·75 | 84·70 | 85·11 |
| 4·80 | 14·28 | 0·230 | 0·045 | 1·18 | 7·96 | 0·24 | 84·59 | 83·77 | 82·08 | 82·42 |
| Average for 3 samples | | | | | | | | | | |
| 3·62 | 13·69 | 0·424 | — | — | 7·13 | 0·29 | 77·47 | 77·00 | — | — |
| Human milk | | | | | | | | | | |
| 2·70 | 11·71 | 0·234 | 0·034 | 1·27 | 7·27 | 0·25 | 61·92 | 62·07 | 60·76 | 61·01 |
| 3·20 | 12·33 | 0·274 | 0·034 | 1·53 | 7·11 | 0·18 | 67·17 | 67·48 | 66·20 | 66·45 |
| 3·36 | 13·08 | 0·299 | 0·033 | 1·70 | 7·70 | 0·22 | 71·94 | 72·21 | 70·99 | 71·23 |
| Average for 3 samples | | | | | | | | | | |
| 3·09 | 12·37 | 0·269 | 0·033 | 1·50 | 7·36 | 0·22 | 67·01 | 67·25 | 65·98 | 66·23 |

and highest fat, lactose and protein contents respectively, while the lowest line of the table gives mean values for all forty-five samples. For the other four species, all the results are given.

For all samples for all species, the values listed under formula I were computed from the equation

$$E = 9{\cdot}11F + 37{\cdot}4TN + 3{\cdot}95L,$$

where $E$ is Cal./100 g. of milk, and $F$, $TN$ and $L$ are percentages of fat, total nitrogen and lactose respectively. The term 37·4 is the product of the conversion factor, 6·38 for protein from nitrogen content, and 5·86, the calorific conversion factor for milk protein.

Formula II was the equation

$$E = 9{\cdot}11F + 37{\cdot}4\,(TN - \text{N.P.N.}) + 3{\cdot}95L.$$

The energy estimate was thus corrected by deducting the non-protein nitrogen from the total nitrogen. The distribution of nitrogen within the N.P.N. fraction was assumed to be approximately: amino acid 3·2, ammonia 0·6, creatine 4·0, creatinine 0·6, urea 9·4, uric acid 2·6, and unaccounted 11·6, mg./100 ml. From these values the energy conversion factor for the non-protein nitrogen component was estimated to be approximately $7{\cdot}4 \times \%$ N.P.N. Inclusion of this term in the calculation of gross calorific values gave the results given under formula III in Table 1.

With the exception of human colostrum the mean calorific value, as calculated from formula I, for each group of samples given in Table 1 exceeded the mean determined value. On average the difference was about 2–3% for cow, goat and ewe milks and about 5% for sow milk. For human milk as distinct from colostrum the difference was only about 0·3%. For the milk of the farm animals a closer estimate was obtained by using formula II which on average gave values that were only about 0·3–0·7% too high for the cow, goat and ewe milks and almost 0·5% too low for the sow milk. The inclusion of the estimated non-protein nitrogen energy component in formula III increased the average values by 0·6–1·1% above the mean values directly determined. For human colostrum and milk the values obtained by formula I were much nearer the directly determined values than those obtained by using either formula II or formula III.

It is suggested that until sufficient detailed analyses are available to enable statistical evaluation of the relationship between gross calorific value and milk composition, the following equation be used:

$$E = 9{\cdot}11F + y(6{\cdot}38 \times TN) + 3{\cdot}95L,$$

where $y = 5{\cdot}12$, 5·52, 5·54 and 5·54 for the sow, cow, ewe and goat respectively. These values were obtained from the protein conversion factor, 5·86, given by Andersen (2), by correcting for the average fraction of the total nitrogen of the milk found to be non-protein in nature. Average values for these fractions, obtained from analyses of a considerable number of milks at this Research Station over the past five years, were 12·6, 5·8, 5·4 and 5·5%, respectively, for the sow, cow, ewe and goat.

The discrepancy between observed and corrected calorific values for human colostrum, and possibly human milk, suggests that either the energy conversion factors used are not sufficiently correct for this species, or that some other energy-yielding component may be present. Present results would indicate that for human milk the best estimate of calorific value is obtained using formula I, that is, treating all nitrogenous constituents as being protein.

SUMMARY

The gross energy content of the milk of the cow, sow, ewe, goat and of human milk has been determined by bomb calorimeter, and compared with results obtained using conversion factors and chemical analysis.

Correction for non-protein nitrogen content led to values agreeing within 0·5–1·0% for the milk of all species. For human colostrum other factors may be involved, and a closer estimate of gross energy value of human milk was obtained by treating all nitrogenous constituents as being protein.

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# 710. THE DETERMINATION OF FREE FATTY ACIDS IN MILK

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(With 1 Figure)

In milk freshly drawn from the cow, the free fatty acid concentration is of the order of 0·0004 M, published values expressed as a fraction of the milk fat ranging from 0·001 to 0·02 M (1,2,3). On storage, however, increases can occur which are sufficient to give rise to off flavours and rancidity, particularly under conditions favourable to lipase activity. These conditions include surface denaturation of the fat globule membrane, foaming and prolonged agitation of milk (4). With increasing mechanization in the dairy industry and the use of homogenized milk for human consumption, higher free fatty acid concentrations in milk must be expected. As a preliminary, therefore, to investigating the effects of different types of milking machine and milk treatment on lipolysis, a study has been made of methods for determining the free fatty acid content of milk.

Direct titration of fatty acids in milk is not practicable owing to the presence of lactic and citric acids and to the buffer capacity of milk proteins and inorganic constituents. Surface tension or pH measurements are insufficiently sensitive or specific. For small milk samples separation of cream, followed by churning, is quantitatively difficult and there is also a significant loss, particularly of the lower fatty acids, during churning (5). Various extraction-titration procedures for use with milk and cream have been proposed (3,6,7,8), but few results of recovery experiments have been reported. Johnson & Gould (6), using a mixture of ether and light petroleum (2:3) for extracting milk to which ethanol had been added, obtained recoveries of 6·6, 28, 76 and 87% respectively, for butyric, caproic, capric and oleic acids that had been added in cream. Acidification to pH 2 with 25% (v/v) sulphuric acid gave increased recoveries, values being 31, 72, 102 and 103% respectively for the same acids. Thomas, Nielson & Olson (7) obtained a separation of fat from milk, based on the B.D.I. milk fat determination method of Sager & Sanders (9) using a wetting agent (Triton X-100, a proprietary detergent) in association with a protein-dispersing agent. The fat phase was then dissolved in a mixture of ether and light petroleum for titration with standard alkali. However, no recovery experiments using this method were reported. Frankel & Tarassuk (8) prepared milks containing known concentrations of free fatty acids by dissolving acids in milk fat and homogenizing them in skim-milk. They considered that some of the observed increase in titration in the method of Johnson & Gould (6) when samples were acidified to pH 2 or lower was due to effects other than those of fatty acids. Frankel & Tarassuk, therefore, did not continue to use acidification with sulphuric acid in their method which otherwise resembled that of Johnson & Gould. Using their proposed method in the present work, recoveries of butyric and caproic acids were only 29 and 38% respectively. Using two extractions recoveries

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for mixtures containing myristic, palmitic and stearic acids averaged 95%, but for butyric, caproic and caprylic acid mixtures recovery was only 52%.

Because of the greater organoleptic importance of butyric and other short chain fatty acids, their more quantitative estimation is desirable. Also, with the reduction of sample size from 125 ml. as used by Johnson & Gould to 10 ml. as in the method of Frankel & Tarassuk and in the method proposed in this paper, with corresponding reduction in acid concentrations at titration, the error caused by using phenolphthalein as indicator became much more significant and the value of α-naphtholphthalein was therefore studied.

## DETERGENT METHOD

Recovery experiments were tried using the detergent method of Thomas, Nielson & Olson (7). The tetrapolyphosphate of Sager & Sanders was not available and sodium hexametaphosphate, which Sager & Sanders (9) suggested as an alternative, was used in all experiments. Addition of free fatty acids to milk showed that recovery by this method was not quantitative, 98% of the butyric, 93% of the caproic and 16% of the stearic acid remaining in the aqueous phase.

The pH of the extraction mixture of Triton X-100 and sodium hexametaphosphate was approximately 7. Many attempts to facilitate extraction of fatty acids by lowering the pH of the solution and adding further protein-dispersing salts (tartrates, citrates, salicylates and phosphates) did not give a quantitative separation of the fat phase. Similarly, the use of alternative detergents functional in acidic solutions failed to give satisfactory fat separation. Because we considered that heating the milk with detergent mixture at approximately 100° C. for 15 min. as recommended by Thomas *et al.* might cause some hydrolysis of the milk fat, further work on this method was discontinued.

## EXTRACTION—TITRATION METHOD

### *Extraction of fatty acids*

(1) *Theoretical.* The approximate distribution of fatty acids in milk fat is well known (10). The main constituents of the fat are the even-numbered saturated fatty acids from butyric to arachidic acid and the unsaturated acids oleic and linoleic. The observed increase in free fatty acids in milk on standing is due mainly to the action of lipases on this fat. Since lipases are often relatively non-specific in their substrates, it may be assumed, in the absence of direct experimental evidence, that the acids liberated in lipolysis may be in approximately the same proportions as in milk fat.

Two factors governing the distribution of fatty acids between milk fat and milk plasma are the hydrocarbon chain, which is lipophilic, and the carboxyl group, which is hydrophilic in character. Thus, in the higher fatty acids the lipophilic effect of the long hydrocarbon chain makes extraction in fat solvents reasonably quantitative. Similar considerations explain the effect of varying the ether/light petroleum ratio on the extraction of fatty acids from milk. Light petroleum, and to a slightly lower degree, diethyl ether, are good solvents for lipophilic substances, so that variation of the ratio of these solvents in the extraction mixture has little effect on the extraction of the higher fatty acids. The slightly amphiprotic character of ether, however, becomes important with short chain fatty acids, where the effect of the carbonyl group is of more significance, and enables them

to be extracted. On the other hand the low solubility of carbonyl-containing substances in light petroleum means that with an increasing amount of that solvent in the ether/light petroleum mixture used, the fraction of the lower fatty acids extracted decreases.

Ionization of the carboxyl group increases considerably its hydrophilic nature. Since milk freshly drawn from the cow ranges in pH between 6·5 and 6·7 (11), it follows that in fresh milk fatty acids are present almost entirely as their anions. Acidification of the solution, by repressing the ionization of the fatty acids, increases their ease of extraction, especially for those with shorter chains. At pH 3 about 98% of any fatty acid is present in the unionized form; no gain in extractability is to be expected on adjusting samples to lower pH values.

(2) *Experimental.* Preliminary experiments in which aqueous alcoholic solutions containing fatty acids were extracted with ether/light petroleum mixtures under conditions comparable with those of Frankel & Tarassuk (8) confirmed the general conclusions outlined above, which are also in agreement with earlier observations (6,8).

Milk fat was prepared from fresh creamery butter by melting it below 60° C., removing the clear fat layer and filtering it through Whatman no. 1 filter-paper. Approximately 0·4M solutions of fatty acids were prepared by dissolving weighed amounts of the acids (laboratory reagent grade) in 50 g. portions of milk fat warmed below 55° C. These fats were then emulsified in fresh warmed skim-milk using a hand emulsifier to give a milk containing 4% fat.

The effect of acidification was examined using an ether/light petroleum mixture (1:1), but otherwise adhering to the method as described below. Percentage recoveries were:

| Acid | Butyric | Caprylic | Palmitic |
|---|---|---|---|
| Milk not acidified | 28 | 78 | 77 |
| Milk extracted at pH 3 | 68 | 90 | 90 |

Similarly, using acidification to pH 3 and varying the ether/light petroleum ratio the results shown in Table 1 were obtained.

Table 1. *Percentage recoveries of fatty acids for various solvent mixtures*

| | Ether/light petroleum ratio | | | |
|---|---|---|---|---|
| Acid | 1:3 | 1:1 | 2:1 | 3:1 |
| Butyric | 49 | 68 | 63 | 73 |
| Caproic | 63 | — | 85 | 87 |
| Caprylic | 90 | 90 | 90·5 | 91 |
| Capric | 92 | — | 93 | — |
| Lauric | 93 | — | 93·5 | — |
| Myristic | 97 | — | 95 | 102 |
| Palmitic | 99 | 90 | 96 | 106 |
| Stearic | 100 | — | 97 | — |
| Oleic | 81 | — | 78 | 93 |

### *Titration of fatty acids*

(1) *Theoretical.* Measurements by potentiometric titration, carried out in aqueous alcohol owing to the sparing solubility in water of the higher members of the series, showed that all the fatty acids occurring in milk fat are weak, with dissociation constants, in water, of the order of 1 to $2 \times 10^{-5}$. Appreciable hydrolysis occurs in titration against standard alkali so that solutions are alkaline at the true end-point of the titration.

However, the pH of the end-point is not a constant, but decreases with decreasing concentration according to the equation (12)

$$\text{pH} = \tfrac{1}{2}\text{p}K_w + \tfrac{1}{2}\text{p}K_a + \tfrac{1}{2}\log C,$$

where $K_w$ is the ionic product of water, $K_a$ is the dissociation constant of the weak acid and $C$ is the concentration of the salt formed. Substitution of typical values for butyric acid illustrate the importance of this effect. Thus, the pH at the end point for titration in a volume of 20 ml. varies approximately as follows:

1 ml. 0·2M butyric acid pH 8·4
1 ml. 0·02M butyric acid pH 7·9

For lower concentrations the end-point gradually approaches the limit, pH 7·0. The use of phenolphthalein, p$K$ 9·4 (13), leads to increasingly greater relative errors as solutions become more dilute.

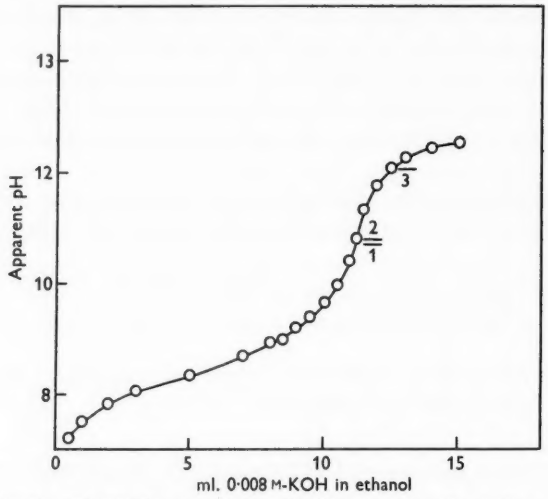


Fig. 1. Potentiometric titration of butyric acid added to fresh milk. 1, True end-point; 2, end-point with α-naphtholphthalein; 3, end-point with phenolphthalein.

(2) *Experimental.* Alcohols resemble water in being amphiprotic solvents, and for the mixed solvent, methanol/ether/light petroleum (3:2:1) used in the proposed method, a similar dilution effect is observed, leading to a similar indicator error. Fig. 1 shows the potentiometric titration curve obtained for a dilute butyric acid solution in the mixed solvent, using a glass electrode, saturated calomel electrode combination. Repeated experiments using 0·1 ml. 1% phenolphthalein and 0·05 ml. 1% α-naphtholphthalein as visual indicators showed that α-naphtholphthalein changes from yellow to greenish yellow close to an apparent pH of 10·7, but that the first colour change for phenolphthalein could not be detected below an apparent pH of about 12·1. For free fatty acid concentrations over the anticipated range 0·5 to 3 mM in milk, the apparent pH at the true end-point would be about 0·4–0·2 pH units lower. In typical recovery experiments using fatty acids added to milk, it was found also that the high carotene concentrations in the milk fat obtained from pasture-fed Jersey cows under New Zealand conditions

rendered the phenolphthalein end-point considerably more difficult to detect. No such difficulty was experienced with α-naphtholphthalein. Slightly steeper potentiometric curves and slightly sharper end-points were obtained using methanol instead of ethanol in the titration medium.

## RECOMMENDED PROCEDURE

In the light of the foregoing results the following method was developed for routine use.

A sample containing 10 ml. milk is pipetted into a 50 ml. centrifuge tube fitted with a ground glass socket. Following the addition of 3 drops of 0·5% bromphenol blue solution, 1N sulphuric acid is added until the colour changes to greenish yellow (usually 1·0–1·5 ml.). Five ml. of 95% neutralized ethanol is added and the tube is stoppered and shaken vigorously for 1 min. Fifteen ml. of a mixture of 2 parts of ether and 1 of light petroleum (B.P. 30/60° C.) is added. The tube is again stoppered and shaken vigorously for 1 min. before being centrifuged at approximately 1500 r.p.m. for 5 min. A 10 ml. portion is withdrawn from the clear supernatant ether layer and added to 10 ml. neutralized methanol in a 100 ml. Erlenmeyer flask for titration against 0·01N alcoholic KOH using α-naphtholphthalein as indicator. The end-point is taken to be the appearance of a greenish tinge in the yellow solution. A blank determination is made using distilled water, reagents and solvents. The difference in titration in ml. multiplied by 1·35 gives directly the concentration, in milliMols, of free fatty acids in the milk. The factor, 1·35, takes account of the observed reduction in volume of the organic phase.

The neutralized methanol and ethanol used above are prepared by the addition of 1% α-naphtholphthalein to the corresponding alcohol to give 0·05 ml. indicator solution in 10 ml., and neutralizing the resulting solution to this indicator using alcoholic KOH. The alcoholic KOH is standardized frequently against weighed quantities of benzoic acid dissolved in the mixed solvent.

### *Recovery experiments*

As indicated in Table 2 for a 2:1 ether/light petroleum mixture recoveries of individual fatty acids, when present in milk at concentrations of what were approximately 0·002M, ranged from 63% for butyric acid to 97% for stearic acid. Similarly, for a mixture of acids in the proportions that are known to exist in typical butterfat (10), and of total fatty acid concentration 0·0016M, the recovery was found to be 83%.

### *Effect of other acids*

Citric acid is normally present in cow's milk at a concentration of 0·2%. Additions of citric acid at concentrations of 0·1, 0·2 and 0·5% failed to yield any measurable increase in titration of the ether/light petroleum extract.

Lactic acid is not normally present in fresh milk (14) and milks containing as much as 0·3% are recognizably sour. Trials with lactic acid added to milk showed very little interference in the proposed method, up to 0·2% lactic acid (difference 0·13 ml. 0·01M-KOH). At higher lactic acid concentrations interference increases rapidly.

Addition of 5N sulphuric acid to milks, to obtain pH values of approximately 3, 2 and 1, gave in repeated experiments no increase in titration of the ether extract between pH 3 and 2, but a possible slight increase at pH 1. Similarly, re-extraction of the aqueous phase

after removal of the ether layer gave, for three fresh milk samples of pH 3, titrations of less than 0·1 ml. 0·01 M-KOH. These results indicate that under the conditions used entrainment of the aqueous phase or shift of phosphate equilibrium, as suggested by Frankel & Tarassuk, is not significant. The results in Table 1, at the highest ether to light petroleum ratio, appear to have been due in part to some factor other than increased recovery of free fatty acids.

## DISCUSSION

The method described in this paper is similar in principle to the solvent extraction procedure of Johnson & Gould but is modified to enable greater recovery of lower fatty acids. It also uses an indicator more appropriate to the end-point of the titration.

The technique used in the recovery experiments of adding the fatty acids direct to the milk fat and then homogenizing the mixture was primarily for convenience of operation since fatty acids above butyric are increasingly less soluble in water, and preparation of appropriate solutions directly in milk becomes increasingly difficult. It has been shown (8) that, at least for butyric acid, no difference in degree of extraction is found between milks rendered rancid by direct addition of fatty acid to the milk or by using solutions of the acid in added fat.

Less well-defined separation of phases and less complete recoveries of fatty acids, especially those of lower molecular weight, were obtained when methanol, instead of ethanol, was added to the aqueous phase.

The importance of the lower fatty acids is shown by the observation (15) that fat obtained by churning rancid milk, although still of a very high free fatty acid content, did not appear rancid, owing to the loss of the water-soluble and flavour-producing free fatty acids.

Repetition of the extraction procedure would increase the amounts of free fatty acids recovered by the present method. However, this has not been attempted because the aim of the investigation was to provide a rapid method for comparison of free fatty acid concentrations in small samples of milk as obtained from large numbers of cows and from different parts of the milking machine. For this purpose the method described above is adequate.

## SUMMARY

An extraction-titration method is described for the determination of free fatty acids in small samples of fresh milk.

Recoveries of fatty acids added to milk ranged from 63% for butyric acid to 97% for stearic acid. For a composite sample approximating milk fat in fatty acid composition the recovery was 83%.

Interference from citric and lactic acids was not significant for reasonably fresh samples.

The method gives a higher recovery of the lower molecular weight fatty acids than is possible by earlier methods.

For titrating very dilute solutions of fatty acids α-naphtholphthalein was shown to be a more accurate indicator than phenolphthalein; it also provides a better defined end-point.

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# 711. SOME FACTORS AFFECTING TURBIMETRIC METHODS FOR THE DETERMINATION OF FAT IN MILK

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(With 3 Figures)

## INTRODUCTION

The possibility of the rapid determination of the fat content of milk from turbidity measurements has often been examined. Since the turbidity of milk is due to light scattered by both the fat globules and the protein micelles, it is necessary to separate these two components of the total turbidity. The component of the turbidity due to the fat globules depends upon both the fat globule size and the fat concentration, so that in principle, turbidity measurements could be used to determine either quantity, provided the experimental conditions were chosen to keep the effects due to the other factor sensibly constant.

The earliest work on this problem appears to have been carried out with simple subjective photometers in the visible region of the spectrum (0·35–0·7 $\mu$). Müller (1) used this method and reported that although the correlation between the opacity and fat content was poor, it was possible to relate the opacity to the mean fat globule size for samples with the same fat content. Later measurements by Schneck (2) using objective photoelectric methods confirmed Müller's observations and provided much preliminary data on the effects of dilution and optical path length. Although the effective wavelength region used by Schneck was not stated, it would appear from the nature of the photocell used to be about 0·45 $\mu$. More recent work by Brio & Vlodavets (3) suggests that when alkali has been added to reduce the turbidity component of the protein, turbimetric methods for fat determination are capable of an accuracy of the order of $\pm 0{\cdot}15\%$.

Turbidity measurements have also been used to determine the changes in fat globule sizes produced by homogenization. Ashworth (4), using a simple colorimeter, found it possible to relate the degree of homogenization to the turbidity at constant fat content. More recently, Lagoni & Merton (5) have developed a spectroturbimetric method for determining the degree of homogenization of milk. Although this method is independent of the fat content, it requires a photometer incorporating a monochromator to enable measurements to be made over a wide range of wavelengths.

It would seem possible to overcome globule size effects by pre-homogenizing to break down the larger fat globules and a patent employing this principle has now been taken out by Batchelor (6). After passing through the homogenizer, the transmission of the milk is measured directly as it passes through a thin absorption cell. No details have been given, however, to indicate the accuracy of this method.

## THEORETICAL

A more detailed treatment of the light scattering by diluted milk suspensions (7) has shown that the experimental results are in quantitative agreement with calculations based on light-scattering theory. Unfortunately, the theory has not yet been completely developed to include more concentrated suspensions where multiple scattering occurs. Most of the considerations discussed here have been derived for dilute suspensions and can therefore be expected to apply only qualitatively to undiluted milk. Nevertheless, these conclusions have been confirmed by the experimental results quoted later.

As indicated earlier, the turbidity, or optical density, of milk depends upon the light scattered by both the fat globules and the colloidal protein. Leviton & Haller (8) have investigated the light scattering by the casein micelles in dilute separated milk and have shown that the turbidity decreases at longer wavelengths in accord with a $\lambda^{-n}$ relationship, where $n$ is approximately 4. In this Tyndall-type of scattering, the radiation is scattered symmetrically about the sample.

The scattering due to the larger fat globules is more complex and has already been discussed in detail (7). In general, it can be seen that the light scattered by the fat globules is much less wavelength-dependent and is radiated more in the forward direction (Mie Effect).

From these considerations, it can be seen that the effects of the radiation scattered by the protein can be considerably reduced by working at longer wavelengths and by collecting only the radiation which is scattered through a small solid angle in the forward direction. The effects of variation in fat globule size are also reduced by working at longer wavelengths, since the smaller globules have a relatively greater effect upon the turbidity at shorter wavelengths; even though their contribution to the total fat volume is small compared with that of the larger globules. It is therefore possible to reduce the effects of protein scattering by purely instrumental factors, as well as by adding alkali.

The main purpose of the present investigation was to compare the accuracy obtainable by the alkali-addition method with that obtainable by the dilution technique at longer wavelengths. In the former method, concentrated milk suspensions were examined in 5 mm. path length cells and the turbidity was measured as the optical density relative to a standard sheet of 'Vitrolite' opal glass. Under these conditions the optical density–fat concentration relationship would not be expected to be linear nor to pass through the origin, owing to multiple scattering effects and the measurement of turbidities relative to a fixed, but unknown standard. The dilution method should follow closely the theoretical treatment however, since true optical densities are measured. Under these conditions a linear relationship passing through the origin would be expected.

## EXPERIMENTAL

For the measurements at a wide band-width with both dilute and undiluted samples, a modified Miller colorimeter was used. Fig. 1 shows a diagram of the essential components of the modified instrument.

The source ($S$) was a 3·5 W. tungsten lamp supplied by a 6 V constant voltage transformer. A heat filter was originally incorporated with the lens $L$, but since the long wavelength transmission limit of this filter was close to 0·7 $\mu$, it was found necessary to remove the filter to enable the instrument to be used at 0·8 $\mu$. After passing through the

sample cell in the 'B' position and the filter *F*, the parallel beam of radiation traversed a blackened tube *T* and reached the 'A' sample position. A Megatron type I.R. barrier-layer photocell (*C*) was placed close to the sample cell as shown. This type of detector had a sensitivity range of about 0·4–0·9 $\mu$, with a maximum sensitivity near 0·75 $\mu$, and was connected directly to a Tinsley mirror galvanometer (*G*) (coil resistance 208 $\Omega$, sensitivity 1540 mm./$\mu$A. at 1 m. scale distance). Using this photocell-galvanometer combination with milk suspensions of known dilutions in the 'B' cell position, it was shown that the response was linear in the optical density range 0–0·7. Measurements were carried out with two filter combinations. Bearing in mind the sensitivity characteristics of the photocell and the emission characteristics of the source, the use of a Kodak 'Wratten' no. 87 filter gave an effective wavelength maximum near 0·8 $\mu$, whilst a combination of the original heat-absorbing filter with an Ilford spectrum orange no. 607 filter provided an effective maximum close to 0·6 $\mu$.

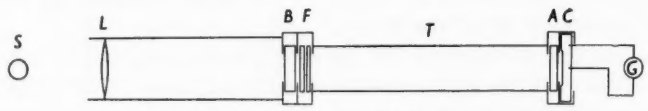


Fig. 1. Modified colorimeter for turbidity measurements.

For measurements by the alkali-addition method (3), 10 ml. of milk were mixed with 1 ml. N sodium hydroxide solution and the sample was examined in a 5 mm. path-length cell in the 'A' position, the optical density being compared against that of a standard sample of 'Vitrolite' opal glass placed in the same cell position. The opal glass standard had previously been ground down to a suitable thickness (about 1 mm.) to ensure that the relative optical densities of the milk samples were in the region 0·3–0·6. In this method, the photocell was placed as close to the sample cell as possible, in order to collect the maximum amount of scattered radiation. Under such conditions, the angle subtended by the detector area at the centre of the sample was very large and amounted to almost 90°.

Dilute suspensions were also examined with wide band-widths, by placing the samples in cells in the 'B' position of the colorimeter. For measurements at narrow band-widths, a modified Unicam SP 500 spectrophotometer was used (7). In both instruments used to examine the dilute suspensions, the angle subtended by the sample at the detector was slightly less than 2°. 1 ml. samples of whole milk were diluted to 500 ml. with water and were examined in 10 mm. path-length glass absorption cells against comparison cells containing water. For separated milk, more concentrated suspensions were used and the turbidities were corrected to the same dilutions as the whole milk suspensions.

The reproducibilities of the turbidities, measured as optical densities, were about $\pm$0·008 for both methods, including the dilution errors.

Fat concentrations were determined by the Gerber method.

## RESULTS

### *Effects of protein turbidity*

With the dilute suspensions, the component of the total turbidity due to the protein was measured at a series of wavelengths with the Unicam Spectrophotometer. These effects were obtained directly by examining the turbidity of separated milk, or indirectly by

comparing the turbidity of diluted whole milk in the presence of dilute alkali with the turbidity in the absence of alkali. The results obtained by the two methods were in good agreement, particularly at the longer wavelengths, and Table 1 demonstrates the decrease in protein scattering at longer wavelengths.

Table 1. *Effects of wavelength on protein turbidity of dilute suspensions*

| Wavelength ($\mu$) | Percentage of total turbidity due to protein |
|---|---|
| 0·4 | 15 |
| 0·6 | 4 |
| 0·8 | 2 |
| 1·0 | 1 |
| 1·2 | 0·7 |

Similar results were obtained with the Miller colorimeter, using the filter combinations corresponding approximately to 0·6 and 0·8 $\mu$.

With the undiluted samples used in the 'A' position with the Miller colorimeter, the relative effects due to protein scattering were much larger, owing to the larger angle of acceptance of the detector and these results are shown in Table 2.

Table 2. *Effects of wavelength on protein turbidity of undiluted milk*

| Effective wavelength ($\mu$) | Percentage of total turbidity due to protein |
|---|---|
| 0·6 | 34 |
| 0·8 | 23 |

The alkali concentration required to reduce the turbidity of the whole milk to a constant value was not found to be very critical, provided a certain minimum amount had been added. An addition of 1 ml. of N sodium hydroxide solution to 10 ml. milk was found to be a convenient amount.

### *Effects of shaking and storage*

Both shaking and storage lead to clumping, or aggregation of the fat globules. Since changes in the fat globule size distribution are known to affect the turbidity, preliminary tests were carried out on both fresh and stored milk samples. It was found necessary to shake the samples vigorously for several minutes before changes in optical density became apparent. Samples which had been allowed to stand overnight at room temperature showed slightly more pronounced effects. Before analysis, the milk samples were mixed by slow inversion of the containers. This gentle mixing was found necessary to ensure that the samples were homogenous, and was insufficient to affect the measured optical densities.

### *Effects of temperature*

The physical state of the fat in the droplets might be expected to influence the results, owing to the effects of changes in refractive index. In order to minimize such errors, the samples were not cooled after taking them from the cow, and were stored at room temperature for a few hours until required. Under these conditions, it was assumed that the fat was in the supercooled liquid state. Warming to about 60° C. for a few minutes, as in

the Russian method (3), in fact produced no changes in the measured optical densities. The effects of pasteurization with subsequent cooling, were not fully investigated, but the effects did not appear to be very great in the sample examined.

### *Effects of wavelength*

The scatter of points from the alkali-addition method was less at 0·8 $\mu$ than 0·6 $\mu$ as expected. This is in agreement with the earlier observations (3) that the accuracy was improved by the use of a red filter, since such a filter would exclude wavelengths less than about 0·7 $\mu$. It was not possible to make measurements with the Miller colorimeter at wavelengths longer than about 0·8 $\mu$, owing to the insensitivity of the photocell at longer wavelengths.

Measurements on dilute suspensions were made at 0·6 and 0·8 $\mu$ with both instruments and at 1·0 and 1·2 $\mu$ with the Unicam spectrophotometer. Although it was possible to use this instrument at even longer wavelengths by suitable modifications (9), strong water absorption bands prevented measurements from being made at longer wavelengths when using a 10 mm. path sample cell. The scatter of the points was reduced at longer wavelengths as expected, and the least scatter was found at 1·0 and 1·2 $\mu$.

It is possible that the larger slit-widths required at longer wavelengths with the Unicam spectrophotometer introduced an averaging factor due to the wider spread of wavelengths. This is considered to be a minor factor, however, since the data from measurements made on the same dilute suspensions at 0·8 $\mu$ with both instruments gave about the same amount of scatter. The effective band-width of the spectrophotometer was several m$\mu$, whilst that used with the colorimeter was of the order of 100 m$\mu$.

### *Effects due to breed of cow and time of lactation*

Both these factors are known to influence the fat-globule size-distribution and will therefore be expected to affect turbidity measurements. In order to study these two factors more closely, milk samples covering a wide range of fat content were taken from cows of different breeds at known times after the calving date. Separate morning and evening samples were used to enable a wide range of fat concentration to be covered and no significant differences were found between the optical behaviour of the two series of samples taken at the different times.

During the first 2 months of lactation, the mean fat-globule size is considerably larger (10) than it is later in lactation and, in general, this would be expected to produce lower turbidity values. The results confirmed that this was usually the case for dilute suspensions, although in some cases the early lactation samples gave apparently normal values.

Fig. 2 shows the results for dilute suspensions at 1·2 $\mu$ for individual samples classified according to breed. Fig. 3 shows the collected results at 0·8 $\mu$ for the alkali-addition method and also the results at 1·2 $\mu$ for the dilution method. In both Fig. 3 (*a*) and Fig. 3 (*b*), samples taken within 7 weeks of lactation have been omitted. The samples from Jerseys are indicated separately in view of their larger fat globule size. Most of the samples were examined by both methods, but a few extra points have been taken from other series of experiments and are shown only on Fig 3 (*b*).

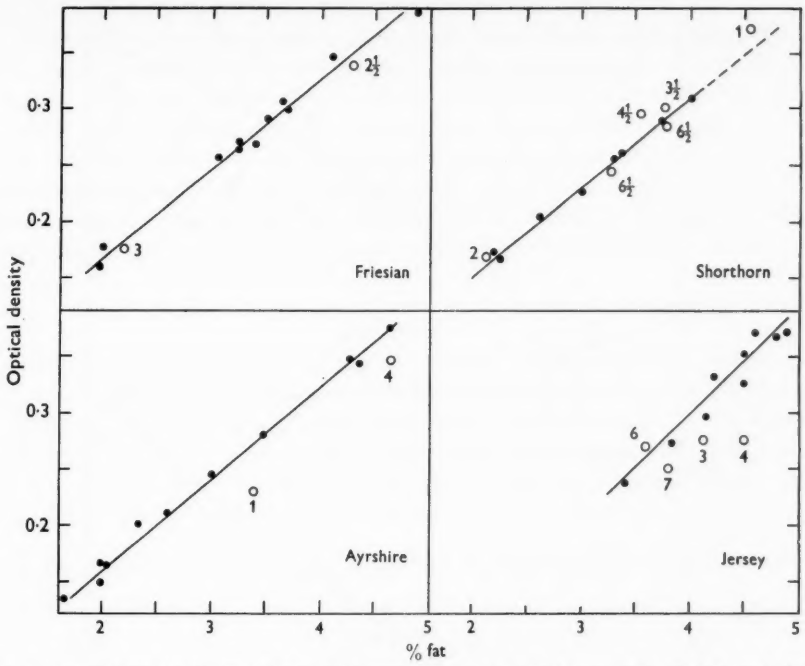


Fig. 2. Optical densities of 1/500 diluted milk suspensions at 1·2 $\mu$ in 10 mm. cells. (Points marked (○) indicate early lactation and the figures indicate the number of weeks after calving.)

## DISCUSSION OF RESULTS

It can be readily seen from Fig. 3 that the dilution method at 1·2 $\mu$ is superior to the alkali-addition method at 0·8 $\mu$. As expected, the results for the dilution method follow a linear relationship passing through the origin. Excluding the early lactation results, the average and maximum deviations of the two methods under different conditions are shown in

Table 3. *Comparison of errors in determination of fat content*

(Omitting samples within 7 weeks of calving date.)

| | Alkali addition method at 0·8 $\mu$ | | Dilution method at 1·2 $\mu$ | |
|---|---|---|---|---|
| | Average deviation | Maximum deviation | Average deviation | Maximum deviation |
| All breeds | ±0·18 | ±0·8 | ±0·08 | ±0·4 |
| All breeds, excluding Jerseys | ±0·15 | ±0·5 | ±0·07 | ±0·2 |
| Friesians | ±0·11 | ±0·2 | ±0·08 | ±0·15 |
| Ayrshires | ±0·13 | ±0·2 | ±0·07 | ±0·15 |
| Shorthorns | ±0·22 | ±0·4 | ±0·04 | ±0·10 |
| Jerseys | ±0·25 | ±0·4 | ±0·11 | ±0·20 |

Table 3. In evaluating these deviations, the sets of values for individual breeds have been considered separately, although the Friesian, Shorthorn and Ayrshire points fell on almost exactly the same experimental curves.

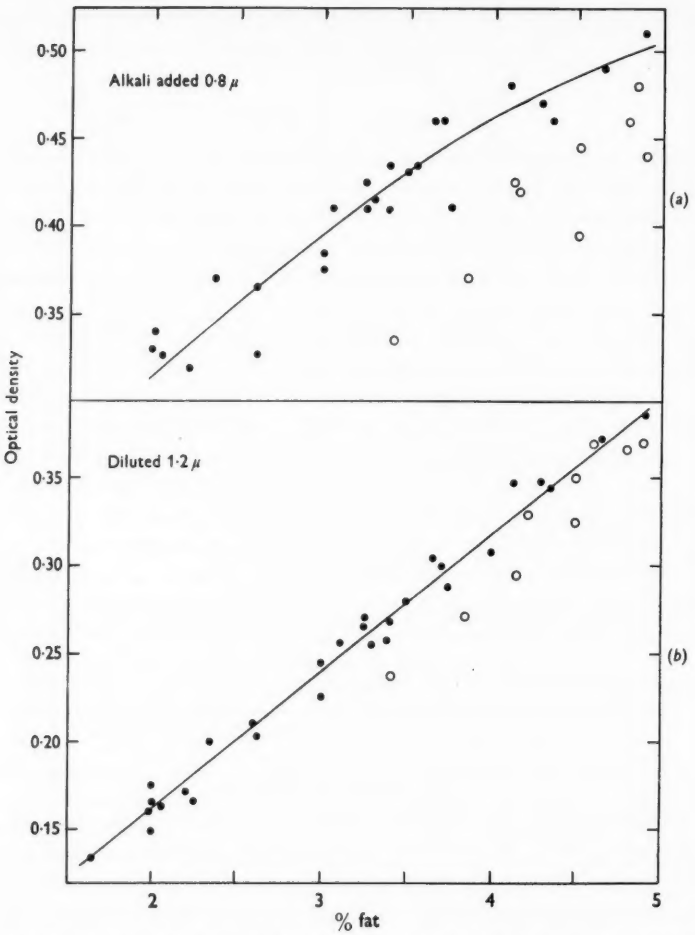


**Fig. 3.** Optical densities of samples from all breeds, excluding early lactation samples. (*a*) Alkali-addition method at 0·8 μ. (*b*) Dilution method at 1·2 μ. ○, Jerseys; ●, other breeds.

All the above results have been taken for single determinations on the same sample, and it is possible that multiple determinations on each sample would have reduced the errors slightly.

## CONCLUSIONS

As can be seen from the above results, the dilution method at longer wavelengths is capable of greater accuracy than the alkali-addition method. Since the experimental error of ±0·008 in optical density determination corresponds to ±0·10% in fat concentration, the dilution method is capable of an accuracy approaching the experimental limit, when applied to samples from a single breed of cow in all except the earliest stages of lactation. Whilst it is difficult to compare the present results with those of previous workers using the alkali-addition method (3), it would seem that accuracy is of the same

order, assuming that the 25% of the samples not considered in their statistical analysis arose from the effects of different breeds and lactation times.

It is difficult to assess the value of this method of analysis for the fat content of bulked milk samples, since the accuracy will be influenced by the composition of the milking herd in relation to both the number of cows in early stages of lactation, and the proportion of cows of the Jersey or Guernsey types. It is unlikely that an accuracy of better than 0·1% will be obtained under the best of conditions, and allowing for all breeds of cows in different stages of lactation, the dilution method may give rise to errors of up to 0·5% with some samples. In view of these limitations, it is considered that the method in its present form is unlikely to prove of value for the routine determination of fat in milk.

SUMMARY

A method for the determination of the fat content of milk from measurements of the turbidities of diluted milk suspensions in the near infra-red region has been shown to provide greater accuracy than the alkali-addition method in the visible region. Variations in results due to breeds of cows and lactation times have been studied, and excluding early lactation samples, the maximum deviation within a given breed was found to correspond to $\pm 0{\cdot}2\%$ in the fat concentration.

Thanks are due to Dr S. J. Rowland for his interest and advice during the course of this work.

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# 712. THE RELATION BETWEEN THE CHEMICAL COMPOSITION OF MILK AND THE STABILITY OF THE CASEINATE COMPLEX

## I. GENERAL INTRODUCTION, DESCRIPTION OF SAMPLES, METHODS AND CHEMICAL COMPOSITION OF SAMPLES

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(With 4 Figures)

### GENERAL INTRODUCTION

The stability of milk protein may be defined as the ability of the casein to remain in colloidal suspension and of the lactalbumin and lactoglobulin to remain in solution when milk is subjected to heat or other modifying influences. A study of the many aspects of protein stability, in addition to being of purely chemical interest, is of practical importance. The successful manufacture of evaporated milk, sweetened evaporated milk (condensed milk) and cheese for example, is largely dependent on the behaviour of the milk proteins, especially casein. The stability to heat of the protein in unconcentrated milk is also becoming an increasingly important factor in milk processing because of the introduction of very high temperature–short time heating to obtain sterile and 'sterilized' milk with an unimpaired flavour.

Although empirical methods have been devised to overcome some of the difficulties in the processing of milk, such as excessive protein-coagulation during the sterilization of evaporated milk, the age-thickening of condensed milk and slow or incomplete coagulation with rennet, the underlying causes of these defects are far from being completely understood. That this should be so despite intensive research is not surprising in view of the variability in the composition of milk and the complex nature of some of its constituents. Since the concentration of milk results in a product with an even more complex composition, it is only reasonable that an attempt should be made to determine the causes of the variation in the stability of the protein in unconcentrated milk before proceeding to the problems connected with concentrated milk products. The attempts that have been made to relate the composition of unconcentrated milk to the stability of the protein to heat and to coagulating agents such as rennet and ethanol have had only limited success.

The exact role of lactalbumin and lactoglobulin in the coagulation of milk is not known but apart from colostrum, whose content of these heat-sensitive proteins is very high, the stability of the major protein, casein, is thought to determine the coagulability of milk. The calcium caseinate in milk, or more precisely the mixture of $\alpha$-, $\beta$- and $\gamma$-caseinates, is combined or associated with varying amounts of inorganic material thought to be mainly tricalcium phosphate. The calcium caseinate–calcium phosphate mixture or complex is colloidally dispersed as globular micelles ranging in diameter from a few millimicrons to 800 m$\mu$, but with a diameter of about 100 m$\mu$ being the most common. It is generally

accepted that this protein dispersion remains stable by virtue of the hydration and negative electrical charge, i.e. the electrokinetic ($\zeta$, zeta) potential, of the micelles. A progressive decrease in either causes the micelles to aggregate and eventually to appear as clots of coagulated protein. It seems probable that one or both of these changes must precede coagulation whatever the coagulating agent. In addition, during the processing of milk the structure of the casein molecule is usually altered before coagulation occurs. For example, coagulation by heat is preceded by some dephosphorylation and prior to coagulation by rennet, casein undergoes a small amount of proteolysis. The stability of the caseinate complex in milk will therefore depend not only on the initial physical and chemical composition of the milk, but in some instances also on the rate or extent of secondary reactions induced by the coagulating agent.

Recent work has suggested that the concentration of ionized calcium and the amount of calcium phosphate associated with the calcium caseinate are related to the colloidal stability of the complex but the results are by no means conclusive. This may have resulted from the inadequacy of existing analytical methods or from the fact that in any one investigation only a small number of the many compositional variables in milk, which might conceivably affect stability, has been determined. Another possible reason is that each milk sample is a unique colloidal system and that therefore no general relation between composition and stability can be arrived at. However, there is no justification as yet for resorting to the latter hypothesis. There is need for an investigation in which milks of widely differing stability to several coagulating agents are analysed in detail and an attempt made to relate stability to chemical composition. This was the object of the present investigation. The coagulating agents used were ethanol, rennet and heat, since these are of considerable practical importance. To ensure a wide variation in stability and chemical composition, the milk samples examined were taken from individual cows in different stages of lactation and from cows with subclinical mastitis as well as from herd bulk milk. The relationships between chemical composition and stability to ethanol, rennet and heat are described respectively in Parts II, III and IV.

## DESCRIPTION OF SAMPLES

The samples, which numbered 132, were obtained from the Hannah Institute herd of pedigree Ayrshire cows. Most of the samples (104) were from forty-two individual cows, some of which were sampled fairly frequently during one lactation, and the remainder were samples of the herd bulk milk. The individual cow samples were taken from cows differing in age and stage of lactation and some were taken from cows with subclinical mastitis. All samples from individual cows were taken from the complete morning milking and the herd bulk samples were obtained by mixing representative samples from the 10-gallon cans containing the complete morning yield of milk from the herd. The volume of each sample taken for examination was about 1500 ml.

### *Herd bulk milk*

Samples of herd bulk milk were taken each month over a period of one year. A total of twenty-eight samples was obtained. When several samples were taken in one month, the composition and stability of each were found to be similar and the results were therefore averaged. In this way, one set of values for each month was obtained for the

composition and stability of the herd bulk milks. At each sampling of the herd bulk milk a note was taken of the number of lactating cows, the stage of lactation of each and the total volume of milk, and from these the mean values were calculated for each month (Table 1). From November to February the bulk milk was provided mainly by cows in the first third of their lactations, and during July, August and September mainly by cows that were well advanced in lactation. The cows were turned out to grass in early May and were returned to the byre in late October.

Table 1. *Monthly herd averages for the number of lactating cows, the average stage of lactation of the cows and the morning yield of milk*

| Month | No. of lactating cows | Average stage of lactation (days) | Morning milk yield of herd (gal.) |
|---|---|---|---|
| January | 32 | 99 | 57 |
| February | 33 | 113 | 47 |
| March | 48 | 120 | 76 |
| April | 48 | 139 | 71 |
| May | 52 | 152 | 77 |
| June | 50 | 176 | 67 |
| July | 38 | 205 | 39 |
| August | 29 | 206 | 30 |
| September | 26 | 186 | 25 |
| October | 31 | 136 | 35 |
| November | 34 | 88 | 50 |
| December | 35 | 89 | 50 |

*Early lactation milk*

The fifteen samples of early lactation milk were obtained from thirteen cows that had been in milk for 10 days or less. These samples were separated into two groups designated colostrum and post-colostrum milk. The colostrum samples were generally easily recognized by their physical appearance, but in doubtful cases chemical composition, e.g. lactose content, was used to assist in their separation from the post-colostrum milks. The colostrum samples were obtained from cows that had been in milk for an average of 4 days and the post-colostrum samples from cows that had been in milk for an average of 8 days.

*Mid-lactation milk*

The fifty-five samples in this group were from twenty-seven cows whose stage of lactation ranged from 16 to 281 days after calving; they represented the period excluding the first 5% and the last 10% of lactation. Thirty-one of these samples were obtained from six cows at intervals during their lactations. All the mid-lactation milks were from cows yielding more than 10 lb. of milk on the morning of sampling.

*Late lactation milk*

The fifteen samples in this group were from thirteen cows in the last 10% of their lactation; the cows had been in milk for periods ranging from 185 to 336 days. The yield of milk on the morning of sampling was less than 6 lb. in eleven instances and ranged from 6·5 to 8·25 lb. in the remaining four.

*Subclinical mastitis milk*

The nineteen samples of subclinical mastitis milk were obtained from thirteen cows. The evidence that these cows had subclinical mastitis was provided by the Veterinary

Pathologist at the Institute, Dr P. S. Blackburn, M.R.C.V.S., who determined the differential cell count of the milks by a technique recently described (1) and also examined them for streptococci and staphylococci associated with mastitis. Seventeen of the subclinical mastitis milks were from cows in mid-lactation and the remaining two were from two cows in early lactation; the chemical composition of the latter samples appeared to be related more to stage of lactation than to the effect of subclinical mastitis. No attempt was made to grade the severity of infection of the thirteen cows.

All the samples of milk from individual cows were examined by Dr Blackburn to ensure that none from cows with subclinical mastitis was included in the other groups of milks.

## METHODS

Analysis of the samples was started only a few hours after they had been taken. The stability tests were done during the forenoon at approximately the same time each day. The chemical analyses were either completed on the day the samples were collected or taken to a stage where ageing would have no effect on the results.

### *Preparation of the samples*

Preliminary studies showed that the removal of the fat from milk had only a small effect on the stability of the milk protein as measured by the strength of ethanol required to cause coagulation and the rate of coagulation by rennet and heat. These observations are in agreement with those of Holm, Deysher & Evans (2), Webb & Holm (3) and Mitamura (4). As the removal of the fat made it slightly easier to detect the moment of coagulation of the milk in the heat stability test and obviated the determination of lipid phosphorus, separated milk was used in all the stability tests and chemical analyses reported in these papers (Parts I–IV).

The fat was separated by centrifuging the whole milk for 30 min. at a force of 1000 *g*. A hole was made in the layer of fat, a glass tube passed through and the separated milk removed by suction. Most of the small amount of sediment in the centrifuge tubes was removed with the separated milk, even although its exclusion did not alter the concentrations of casein, calcium and phosphorus in the milk; the sediment was presumably mainly cellular debris. The separated milk obtained by this procedure contained about 0·1% fat as determined by the Röse-Gottlieb method but, as a check, all samples were tested by the Gerber method to ensure that this level was not exceeded. The temperature of the separated milk was usually approximately 20° C. but when not, it was adjusted to this temperature for the analysis.

### *Protein stability tests*

*Ethanol.* The stability of milk protein to ethanol was determined by finding the strength of ethanol solution which, when added to an equal volume of milk, caused the formation of clots. Thirteen aqueous solutions of ethanol were used covering the range 66–90% (v/v) ethanol in 2% intervals. The procedure was as follows: 2 ml. of milk were pipetted into a test-tube, 2 ml. of 90% ethanol added and the liquids mixed by gentle inversion. The mixture was poured into a glass Petri dish and examined for the presence of clots. The test was repeated with the other ethanol solutions, in order of decreasing strength

until coagulation did not occur. The strength of the weakest ethanol solution that caused coagulation was recorded.

*Rennet.* The stability of milk protein to rennet was determined by measuring the time required for clots to appear in milk to which a small volume of a dilute rennet solution had been added. The temperature of the milk was maintained at 30° C. during the test. The method used was that described by Berridge (5). The renneting time of each milk was determined in triplicate to the nearest second; deviation from the mean was rarely more than a few seconds. The mean value was recorded to the nearest 0·1 min. The conditions of the test were such that milk of average composition coagulated in about 4 min.

*Heat.* The stability of milk protein to heat was measured by determining the time required for clots to appear in milk heated at 130, 140 and 150° C. Temperatures of this order were necessary to cause coagulation in a reasonable time and three were used so that the temperature coefficient of the coagulation reaction could be calculated. The procedure was as follows: 1·5 ml. of milk were pipetted into a thick-walled 'Pyrex' tube about 12 cm. long and 3·5 ml. in capacity. The tube was closed with a 'Silicone' rubber stopper and attached horizontally to a metal carriage in such a way that the stopper could not be forced from the tube. The carriage was then immersed in liquid paraffin maintained at one of the prescribed temperatures in a bath equipped with a stirrer, an accurate thermometer and electric heaters controlled by a contact thermometer and an electronic relay. At the moment of immersion of the tube, a stop-watch was started. The carriage was given a gentle rocking motion at a constant speed so that the milk flowed slowly backward and forward from one end of the tube to the other. When clots were seen in the milk, the watch was stopped and the time required for coagulation recorded to the nearest 0·1 min.

The coagulation time of each sample was determined in duplicate at each temperature and the mean calculated. When the coagulation time was short ($< 10$ min.), variation from the mean was negligible. When the coagulation time was longer (10–40 min.), variation from the mean was usually within $\pm 0{\cdot}5$ min. and when very long ($> 40$ min.), or when the coagulation point was difficult to detect, the variation was of the order of $\pm 1$–2 min. A similar technique for assessing the stability of milk to heat has been used by Cole & Tarassuk (6) and Pyne & McHenry (7).

### *Chemical analysis of the samples*

In addition to determining the major constituents, the calcium, magnesium, phosphorus and citric acid were partitioned into their colloidal and soluble fractions, and the nitrogen into the non-protein fraction and the various protein fractions. Values for sodium, potassium and chloride were also obtained and pH and titratable acidity were determined.

The major constituents in each milk sample were determined as follows: water, Method 2 in British Standard 1741 (8); protein (total N × 6·38), micro-Kjeldahl method (9,10); lactose (anhydrous), Rowland's modification (11) of the chloramine-T method of Hinton & Macara (12); ash, British Standard 1741 (8).

The ash was dissolved in dilute acid and the solution analysed for sodium and potassium with a flame-photometer. Chloride was estimated by the method of Davies (13) and pH measured with a glass electrode. Titratable acidity was determined as described in British Standard 1741 (8), but the results were expressed as ml. of 0·1 N sodium hydroxide solution per 100 g. of milk instead of g. of 'lactic acid' per 100 ml. of milk.

The nitrogen in the samples was partitioned into casein N, lactalbumin plus lactoglobulin N, proteose-peptone N and non-protein N by Rowland's method (14), except that tungstic acid was used to prepare the filtrate containing only non-protein nitrogen. The concentration of nitrogen in the various filtrates was determined by a micro-Kjeldahl method based on those of Hiller, Plazin & Van Slyke (9) and McKenzie & Wallace (10).

To partition the calcium, magnesium, phosphorus and citric acid it was necessary to prepare an ultrafiltrate and a trichloroacetic acid (TCA) filtrate from each sample of milk. An ultrafiltrate was obtained by filtering milk through 'Cellophane' in an improved model of the apparatus devised by Clark (15). The details of this procedure and a comparison of values for the soluble constituents of milk obtained by both ultrafiltration and dialysis will be described in another publication. The TCA filtrate was prepared by diluting 20 ml. of milk to 100 ml. with a 15% (w/v) solution of TCA, shaking the mixture vigorously for a few seconds and after 30 min., filtering it through a Whatman no. 40 paper. The ultrafiltrate and TCA filtrate were used as described below.

*The partition and estimation of phosphorus.* Five estimations were made as follows:

(1) Total phosphorus, i.e. colloidal inorganic P + casein P + soluble inorganic P + ester P.

(2) Total acid-soluble phosphorus, i.e. colloidal inorganic P + soluble inorganic P + ester P.

(3) Inorganic acid-soluble phosphorus, i.e. colloidal inorganic P + soluble inorganic P.

(4) Total soluble phosphorus, i.e. soluble inorganic P + ester P.

(5) Soluble inorganic phosphorus.

From these estimations, the concentrations of each of the four principal forms of phosphorus in milk were calculated thus:

$$\text{casein P} = 1 - 2,$$
$$\text{ester P} = 2 - 3 \text{ (or } 4 - 5),$$
$$\text{colloidal inorganic P} = 3 - 5 \text{ (or } 2 - 4),$$
$$\text{soluble inorganic P} = 5.$$

The two methods of calculating the concentrations of ester P and colloidal inorganic P gave similar results. Estimations 1, 2 and 4 were made respectively on samples of milk, TCA filtrate and ultrafiltrate by digestion with perchloric acid and hydrogen peroxide followed by colorimetric estimation of the phosphorus as molybdenum blue. The digestion and colorimetric procedures were based on those of Allen (16). Estimations 3 and 5 were made on samples of TCA filtrate and ultrafiltrate respectively, but without the digestion stage. That no hydrolysis of ester compounds occurred in estimations 3 and 5 was shown by preliminary studies using two alternative procedures in which special precautions are taken to prevent hydrolysis (17, 18).

*The partition and estimation of calcium.* Three estimations were first made as follows:

(1) Total calcium, i.e. colloidal inorganic Ca + caseinate Ca + soluble un-ionized Ca + ionized Ca.

(2) Total soluble calcium, i.e. soluble un-ionized Ca + ionized Ca.

(3) Ionized calcium.

From these estimations, values for the following fractions were calculated:

$$\text{colloidal inorganic Ca + caseinate Ca} = 1 - 2,$$
$$\text{soluble un-ionized Ca} = 2 - 3.$$

Estimations 1 and 2 were made respectively on TCA filtrate and ultrafiltrate by precipitating the calcium as oxalate and titrating the oxalate with potassium permanganate solution. The technique was based on the micro-method of the Association of Official Agricultural Chemists (19). Estimation 3 was made on ultrafiltrate by the colorimetric, murexide (ammonium purpurate) method described by Smeets and Seekles (20,21,22,23), except that in the preparation of the standard solutions for the calibration curve a more alkaline buffer solution was found necessary to achieve the correct pH, and the amount of magnesium in the salt solution used to obtain the correct ionic strength was reduced to allow for the fact that only about one-quarter of the soluble magnesium in milk is ionized (24).

To complete the partition of calcium it was necessary to obtain a value for either colloidal inorganic calcium or caseinate calcium. The first alternative was adopted. The concentration of colloidal inorganic calcium in milk can be calculated from the concentration of colloidal inorganic phosphorus provided the composition of the colloidal calcium phosphate is known. This information was obtained by the method of Pyne & Ryan (25) which determines the relative amounts of colloidal inorganic phosphorus in the forms of $Ca_3(PO_4)_2$ and $Ca_2(HPO_4)_2$. Ultrafiltrate was used in place of the whey or diffusate used by Pyne & Ryan. The concentrations of colloidal tricalcium and dicalcium phosphate were calculated and hence the concentration of calcium in colloidal inorganic form. The concentration of caseinate calcium was then obtained by subtracting the value for colloidal inorganic calcium from the value for 1 – 2 above. In this way, the concentration of each of the four principal forms of calcium in milk was obtained.

*The partition and estimation of magnesium.* Magnesium probably exists in milk in forms similar to those in which calcium exists but the lack of a suitable method for determining ionized magnesium made it impossible to achieve a complete partition. This was not considered a serious drawback because the total amount of magnesium in milk is only about 12 mg./100 g. and therefore the amounts occurring in the different forms are relatively small. The following estimations were made:

(1) Total magnesium, i.e. colloidal Mg + soluble Mg.
(2) Soluble magnesium.

The concentration of colloidal magnesium was obtained by difference (1 – 2). Estimation 1 was made on the supernatant liquid after the precipitation of calcium oxalate in the TCA filtrate, and estimation 2 on the supernatant liquid after the precipitation of calcium oxalate in the ultrafiltrate. In each estimation, the magnesium was precipitated as $MgNH_4PO_4.6H_2O$ and the precipitate washed, using techniques based on those of Bushill, Lampitt & Filmer (26) and Michaels, Anderson, Margen & Kinsell (27). The precipitate was dissolved in perchloric acid, the phosphorus in solution estimated as molybdenum blue in the way already described and the equivalent amount of magnesium calculated.

*The partition and estimation of citric acid.* Most of the citric acid in milk is in the aqueous phase but a small amount is associated with the caseinate in the dispersed phase (28,29,30). It is therefore possible to partition the citric acid into soluble and colloidal fractions. Two estimations were made as follows:

(1) Total citric acid, i.e. colloidal citric acid + soluble citric acid.
(2) Soluble citric acid.

The concentration of colloidal citric acid was obtained by difference (1 – 2). Estimation 1 was made on the TCA filtrate and estimation 2 on the ultrafiltrate, by the method of Saffran & Denstedt (31).

In the chemical analysis of the samples, each estimation was made in duplicate and the mean value used in comparing composition and protein stability. All concentrations were expressed as mg. or g. per 100 g. of milk. Where filtrates were used, correction was made for the volume of the precipitate. The use of a TCA filtrate for estimating total calcium and total magnesium was advocated by Sanders (32) as an alternative to a solution of milk ash; tests showed that both methods gave identical results. The concentrations of constituents in ultrafiltrate were determined as mg. per 100 g. of ultrafiltrate and converted to mg. per 100 g. of milk by multiplying by the factor (wt. water in 100 g. milk/wt. water in 100 g. ultrafiltrate). This was usually about 0·965. No allowance was made for the small amount of bound water in milk (33).

## COMPOSITION OF SAMPLES

As the fat was removed from all samples before examination, all results apply to separated milk and not to whole milk.

### *Herd bulk milk (twelve samples)*

The mean values, ranges and standard deviations for the concentrations of the constituents of the herd bulk milks are given in Table 2, together with the values for the constituents of the caseinate complex expressed as mg. per g. of casein. The appreciable differences between the minimum and maximum concentrations of most constituents could be attributed mainly to the fact that the average stage of lactation of the herd ranged from 88 to 206 days (see Table 1). Nevertheless, the standard deviations indicate that most of the herd bulk milks were fairly similar in composition.

The mean values for the various constituents, per 100 g. of milk or per g. of casein, agree reasonably well with those reported in the literature. The mean value for the concentration of ionized calcium (11·4 mg./100 g. milk) is similar to that obtained by Smeet and Seekles (20, 21, 22, 23), whose method was used. By an entirely different method, Christianson, Jenness & Coulter (34) and van Kreveld & van Minnen (24) found the average concentration of ionized calcium in milk to be slightly lower, about 9 mg./100 g. of milk. According to Boulet & Rose (35), the method of Smeets and Seekles would tend to give too high a value for the concentration of ionized calcium because the formation of calcium purpurate would induce dissociation of calcium citrate.

Pyne & Ryan (25) found that on average, 88% of the colloidal inorganic phosphorus in milk was present as tricalcium phosphate. The present results gave an average value of 70% and showed also that the composition of the phosphate varied greatly in different milks. There is still much controversy regarding the composition of the colloidal calcium phosphate, and recent work of Evenhuis (36) suggests that it may be entirely in the tricalcic form. Since the present estimates of the percentage of colloidal inorganic phosphorus existing as tricalcium phosphate were lower than those of Pyne & Ryan, and considerably less than those of Evenhuis, it is possible that our values for colloidal calcium phosphate (mg./g. casein) are slightly low and the values for caseinate calcium consequently slightly high. However, the average values for these two constituents (mg./g.

Table 2. *Composition of fat-free, herd bulk milk (twelve samples)*

| | Mean | Range | Standard deviation |
|---|---|---|---|
| | (g./100 g. milk) | | |
| Total solids | 9·07 | 8·82–9·30 | 0·14 |
| Protein (T.N. × 6·38) | 3·30 | 3·04–3·54 | 0·16 |
| Lactose (anhydrous) | 4·70 | 4·50–4·91 | 0·13 |
| Ash | 0·76 | 0·73–0·78 | 0·02 |
| pH | 6·72 | 6·65–6·84 | 0·08 |
| Titratable acidity (ml. 0·1 N-NaOH/100 g. milk) | 15·5 | 12·2–17·1 | 0·17 |
| | (mg./100 g. milk) | | |
| Total calcium | 117·7 | 110·9–120·3 | 2·5 |
| Total magnesium | 12·1 | 11·4–13·0 | 0·6 |
| Total citric acid | 176 | 166–192 | 9 |
| Total phosphorus | 95·1 | 79·8–101·7 | 6·1 |
| Sodium | 58 | 47–77 | 10 |
| Potassium | 140 | 113–171 | 14 |
| Chloride | 104·5 | 89·8–127·0 | 11·4 |
| Casein N | 402·3 | 380·0–424·2 | 15·6 |
| Lactalbumin + lactoglobulin N | 65·9 | 55·6–73·9 | 5·9 |
| Proteose–peptone N | 24·6 | 19·4–30·6 | 4·0 |
| Non-protein N | 23·8 | 18·5–33·0 | 4·6 |
| Colloidal inorganic Ca | 49·7 | 41·8–54·0 | 3·2 |
| Caseinate Ca | 31·4 | 28·9–33·9 | 1·7 |
| Soluble un-ionized Ca | 25·3 | 21·9–28·8 | 1·9 |
| Ionized Ca | 11·4 | 10·5–12·8 | 0·7 |
| Colloidal Mg | 4·3 | 3·8–4·5 | 0·2 |
| Soluble Mg | 7·8 | 7·0–8·5 | 0·4 |
| Colloidal citric acid | 19 | 15–22 | 2·6 |
| Soluble citric acid | 158 | 143–175 | 11 |
| Colloidal inorganic P | 29·3 | 24·9–31·1 | 1·6 |
| Casein P | 21·5 | 18·7–23·0 | 1·3 |
| Soluble inorganic P | 33·6 | 27·0–38·9 | 3·7 |
| Ester P | 10·6 | 7·7–13·1 | 1·7 |
| | (mg/g. casein) | | |
| Caseinate calcium | 12·2 | 11·1–13·9 | 0·8 |
| Casein phosphorus | 8·4 | 7·7–8·9 | 0·4 |
| Colloidal inorganic calcium | 19·3 | 17·1–20·3 | 0·9 |
| Colloidal inorganic phosphorus | 11·4 | 10·2–12·2 | 0·6 |
| Tricalcium phosphate | 39·1 | 33·3–47·1 | 4·6 |
| Dicalcium phosphate | 16·1 | 9·1–20·6 | 4·0 |
| Tri- plus dicalcium phosphate | 55·2 | 49·0–58·9 | 2·7 |
| Colloidal magnesium | 1·7 | 1·5–1·8 | 0·1 |
| Colloidal citric acid | 7·3 | 5·5–8·4 | 0·9 |

casein) in milk of normal average composition (Tables 2 and 4) are similar to the corresponding values that can be calculated from the data of Ramsdell & Whittier (37) on the composition of the caseinate complex obtained by centrifuging milk.

*Early lactation milk (fifteen samples)*

The mean composition of the two groups of early lactation milk is shown in Table 3, together with the ranges of values. The standard deviations were not calculated because the number of samples in each group was small but the mean values have been expressed as percentages of the corresponding herd bulk means for comparison. There was considerable variation in the composition of the colostrum samples but all differed from the herd bulk milks in being richer in most constituents, especially ester phosphorus, lactalbumin plus lactoglobulin, ionized calcium and non-protein nitrogen. The high average concen-

Table 3. *Composition of fat-free, early lactation milk (fifteen samples) with the mean value for each constituent expressed as a percentage of the corresponding herd bulk mean value*

| | Colostrum group (seven samples) | | | Post-colostrum group (eight samples) | | |
|---|---|---|---|---|---|---|
| | Mean (g./100 g. milk) | Range (g./100 g. milk) | Mean as % of herd bulk mean | Mean (g./100 g. milk) | Range (g./100 g. milk) | Mean as % of herd bulk mean |
| Total solids | 9·82 | 8·90–11·56 | 108 | 9·92 | 9·54–10·17 | 109 |
| Protein (T.N. × 6·38) | 4·39 | 3·48–6·37 | 133 | 3·98 | 3·67–4·45 | 121 |
| Lactose (anhydrous) | 4·20 | 3·82–4·46 | 89 | 4·78 | 4·50–5·09 | 102 |
| Ash | 0·85 | 0·80–0·89 | 112 | 0·82 | 0·76–0·89 | 108 |
| pH | 6·53 | 6·41–6·62 | — | 6·55 | 6·47–6·69 | — |
| Titratable acidity (ml. 0·1 N-NaOH/100 g. milk) | 21·5 | 19·0–25·0 | 139 | 21·6 | 20·2–22·9 | 140 |
| | (mg./100 g. milk) | | | (mg./100 g. milk) | | |
| Total calcium | 130·6 | 116·4–150·7 | 111 | 134·5 | 124·9–141·7 | 114 |
| Total magnesium | 13·6 | 12·2–15·4 | 112 | 13·0 | 11·7–14·8 | 107 |
| Total citric acid | 176 | 126–236 | 100 | 185 | 145–238 | 105 |
| Total phosphorus | 113·7 | 104·0–124·8 | 120 | 117·5 | 107·4–127·1 | 124 |
| Sodium | 67 | 46–78 | 116 | 57 | 42–78 | 98 |
| Potassium | 160 | 146–173 | 114 | 164 | 145–182 | 117 |
| Chloride | 127·7 | 110·0–151·6 | 122 | 84·6 | 67·4–104·2 | 81 |
| Casein N | 461·0 | 382·7–529·0 | 115 | 489·0 | 458·2–530·8 | 122 |
| Lactalbumin + lactoglobulin N | 163·4 | 102·8–442·1 | 248 | 87·9 | 72·5–113·8 | 133 |
| Proteose–peptone N | 28·4 | 14·8–48·1 | 115 | 19·2 | 14·1–28·0 | 78 |
| Non-protein N | 35·2 | 27·2–44·1 | 148 | 27·1 | 24·9–32·0 | 114 |
| Colloidal inorganic Ca | 44·7 | 34·8–56·8 | 90 | 51·0 | 42·7–61·3 | 103 |
| Caseinate Ca | 35·5 | 28·6–42·2 | 113 | 36·4 | 32·6–39·2 | 116 |
| Soluble un-ionized Ca | 33·4 | 26·8–41·8 | 132 | 31·5 | 25·3–37·7 | 124 |
| Ionized Ca | 17·0 | 15·1–20·1 | 149 | 15·6 | 13·1–17·7 | 137 |
| Colloidal Mg | 4·4 | 3·5–5·8 | 102 | 4·3 | 3·7–4·8 | 100 |
| Soluble Mg | 9·2 | 8·0–10·9 | 118 | 8·8 | 7·7–10·4 | 113 |
| Colloidal citric acid | 18 | 11–25 | 95 | 18 | 10–25 | 95 |
| Soluble citric acid | 158 | 109–216 | 100 | 167 | 122–216 | 106 |
| Colloidal inorganic P | 27·2 | 20·4–34·6 | 93 | 29·1 | 23·2–34·9 | 99 |
| Casein P | 24·3 | 20·3–28·6 | 113 | 24·8 | 22·4–26·9 | 115 |
| Soluble inorganic P | 31·7 | 26·9–38·2 | 94 | 37·1 | 33·0–41·3 | 110 |
| Ester P | 30·5 | 25·0–41·2 | 288 | 26·5 | 24·6–29·6 | 250 |
| | (mg./g. casein) | | | (mg./g. casein) | | |
| Caseinate calcium | 12·2 | 9·9–16·0 | 100 | 11·7 | 10·9–12·9 | 96 |
| Casein phosphorus | 8·3 | 7·6–9·4 | 99 | 8·0 | 6·9–8·7 | 95 |
| Colloidal inorganic calcium | 15·4 | 10·9–21·5 | 80 | 16·4 | 12·9–20·1 | 85 |
| Colloidal inorganic phosphorus | 9·4 | 6·4–13·1 | 82 | 9·3 | 7·0–11·4 | 82 |
| Tricalcium phosphate | 28·0 | 16·6–38·7 | 73 | 37·0 | 29·4–45·4 | 95 |
| Dicalcium phosphate | 16·8 | 8·4–24·9 | 104 | 8·6 | 2·1–12·8 | 53 |
| Tri- plus dicalcium phosphate | 44·8 | 31·0–62·4 | 81 | 45·6 | 34·8–55·9 | 83 |
| Colloidal magnesium | 1·5 | 1·1–1·8 | 88 | 1·4 | 1·2–1·6 | 82 |
| Colloidal citric acid | 6·2 | 3·1–9·5 | 85 | 5·8 | 3·6–7·6 | 79 |

tration of proteose-peptone nitrogen was caused by two samples that contained respectively 41·6 and 48·1 mg./100 g. of milk; the average value for this constituent in the other five samples was 21·9 mg./100 g. of milk, corresponding to 89% of the herd bulk mean. These five samples, like the post-colostrum samples, therefore had a content of proteose-peptone below average. The average concentrations of colloidal inorganic calcium and colloidal inorganic phosphorus were lower than in the herd bulk milk. This difference, coupled with the higher casein content of colostrum, meant that the average amount of colloidal calcium phosphate associated with 1 g. of casein in the colostrum samples was lower than in the herd bulk milks; the decrease appeared to be solely in the tricalcium phosphate fraction. The decrease was probably linked with the lower pH values of the

colostrum samples. The average amounts of colloidal magnesium and citric acid associated with casein were also less in the colostrum samples than in the herd bulk samples.

The post-colostrum samples differed from the herd bulk milks in much the same way as the colostrum samples but to a lesser degree. The average concentrations of ester phosphorus, ionized calcium and lactalbumin plus lactoglobulin were still appreciably above the corresponding herd bulk averages, but the average concentration of lactalbumin plus lactoglobulin in the post-colostrum samples was only about half that of the colostrum samples. The higher average concentration of casein in colostrum compared with herd bulk milk was maintained and even exceeded in the post-colostrum milks. As in the colostrum samples, the amount of colloidal material associated with 1 g. of casein was less than in the herd bulk milks and in this respect there was little difference between colostrum and post-colostrum milk except that in the latter, the decrease in colloidal calcium phosphate seemed to be mainly in the dicalcium phosphate fraction. The average concentration of lactose in the post-colostrum milks reached the level observed for the herd bulk milks, and the sodium and chloride contents were less than those of the colostrum samples. The average pH values and titratable acidities of the colostrum and post-colostrum milks were similar and showed the greater acidity of these samples compared with that of the herd bulk milks.

*Mid-lactation milk* (*fifty-five samples*)

As would be expected, the mean composition of the mid-lactation samples was similar to that of the herd bulk milks but the variation in composition between individual samples was much greater (Table 4). This wide variation could be attributed to the fact that, although designated as mid-lactation milk, the samples came from twenty-seven cows whose stage of lactation ranged from 16 to 281 days after calving. The composition of the caseinate complex in these milks showed much variation, especially in the amount and composition of the colloidal calcium phosphate, and in the amount of citric acid, associated with 1 g. of casein.

*Late lactation milk* (*fifteen samples*)

The mean composition of these milks (Table 5) differed considerably from the mean composition of the herd bulk milks. On average, the late lactation milks contained more protein, especially proteose-peptone and lactalbumin plus lactoglobulin, and less lactose, and had a higher pH and a much lower titratable acidity than the herd bulk milks. They were also richer in sodium and chloride but poorer in potassium. The concentrations of the soluble fractions of calcium, citric acid and especially of phosphorus were low, whereas the concentrations of the colloidal fractions of these constituents and of magnesium were high in the late lactation milks. The casein in these milks had an increased amount of calcium, inorganic phosphorus and magnesium associated with it despite the greater concentration of the casein.

*Subclinical mastitis milk* (*nineteen samples*)

The mean composition of the milks from cows with subclinical mastitis is shown in Table 6. In compiling this table, the results for the two samples from cows that had been lactating for only 6 and 9 days respectively were omitted.

Table 4. *Composition of fat-free, mid-lactation milk (fifty-five samples) with the mean value for each constituent expressed as a percentage of the corresponding herd bulk mean value*

| | Mean | Range | Standard deviation | Mean as % of herd bulk mean |
|---|---|---|---|---|
| | (g./100 g. milk) | | | |
| Total solids | 9·07 | 8·35–9·97 | 0·34 | 100 |
| Protein (T.N. × 6·38) | 3·30 | 2·63–3·83 | 0·26 | 100 |
| Lactose (anhydrous) | 4·74 | 4·40–5·12 | 0·18 | 101 |
| Ash | 0·75 | 0·66–0·83 | 0·04 | 99 |
| pH | 6·73 | 6·53–6·86 | 0·08 | — |
| Titratable acidity (ml. 0·1 N-NaOH/100 g. milk) | 16·0 | 11·5–20·0 | 2·34 | 103 |
| | (mg./100 g. milk) | | | |
| Total calcium | 115·3 | 91·7–136·3 | 10·4 | 98 |
| Total magnesium | 11·8 | 9·6–14·7 | 1·3 | 98 |
| Total citric acid | 166 | 108–230 | 28 | 94 |
| Total phosphorus | 95·2 | 69·0–119·6 | 12·8 | 100 |
| Sodium | 56 | 32–80 | 12 | 97 |
| Potassium | 154 | 123–176 | 14 | 110 |
| Chloride | 103·5 | 59·7–149·8 | 16·8 | 99 |
| Casein N | 404·5 | 309·4–493·1 | 36·2 | 101 |
| Lactalbumin + lactoglobulin N | 64·5 | 43·4–82·5 | 8·8 | 98 |
| Proteose–peptone N | 23·7 | 13·8–40·5 | 5·9 | 96 |
| Non-protein N | 23·9 | 17·6–32·4 | 4·0 | 100 |
| Colloidal inorganic Ca | 47·7 | 27·5–64·2 | 9·0 | 96 |
| Caseinate Ca | 30·4 | 23·0–37·5 | 3·6 | 97 |
| Soluble un-ionized Ca | 25·6 | 16·2–39·0 | 5·2 | 101 |
| Ionized Ca | 11·6 | 9·2–14·0 | 1·2 | 102 |
| Colloidal Mg | 4·1 | 2·7–5·8 | 0·7 | 95 |
| Soluble Mg | 7·7 | 5·9–9·6 | 0·9 | 99 |
| Colloidal citric acid | 17 | 1–57 | 9 | 90 |
| Soluble citric acid | 149 | 94–201 | 27 | 94 |
| Colloidal inorganic P | 27·9 | 16·1–38·1 | 5·1 | 95 |
| Casein P | 21·4 | 16·7–26·5 | 2·3 | 100 |
| Soluble inorganic P | 35·5 | 20·3–52·4 | 7·0 | 106 |
| Ester P | 10·5 | 2·9–19·5 | 3·9 | 99 |
| | (mg./g. casein) | | | |
| Caseinate calcium | 11·9 | 9·0–16·1 | 1·3 | 98 |
| Casein phosphorus | 8·3 | 7·0–10·2 | 0·6 | 99 |
| Colloidal inorganic calcium | 18·6 | 11·4–25·4 | 3·4 | 96 |
| Colloidal inorganic phosphorus | 10·9 | 6·5–12·3 | 2·0 | 96 |
| Tricalcium phosphate | 38·1 | 17·8–54·7 | 8·2 | 98 |
| Dicalcium phosphate | 14·5 | 0·0–25·9 | 5·9 | 90 |
| Tri- plus dicalcium phosphate | 52·6 | 32·3–71·8 | 9·7 | 95 |
| Colloidal magnesium | 1·6 | 1·1–2·1 | 0·7 | 94 |
| Colloidal citric acid | 6·7 | 0·3–24·9 | 3·9 | 92 |

Table 6 shows that the subclinical mastitis milks were low in total solids mainly as a result of low lactose contents. To compensate osmotically for this, the concentrations of sodium and chloride were high. The average nitrogen partition in these milks was appreciably different from that of the herd bulk milks; the concentration of casein was lower and the concentration of serum proteins was higher. The subclinical mastitis milks contained less citric acid and phosphorus than the herd bulk milks. The decrease in phosphorus was mainly in the soluble fractions, whereas the concentrations of both colloidal and soluble citric acid were low. The mean values for colloidal inorganic calcium and phosphorus were similar to those of the herd bulk milk, but the lower values for casein meant that the amounts of these constituents associated with 1 g. of casein were greater than in the herd bulk milks. In these respects, the average composition of the caseinate complex in the subclinical mastitis milks was similar to that in the late lactation milks.

Table 5. *Composition of fat-free, late lactation milk (fifteen samples) with the mean value for each constituent expressed as a percentage of the corresponding herd bulk mean value*

| | Mean | Range | Standard deviation | Mean as % of herd bulk mean |
|---|---|---|---|---|
| | (g./100 g. milk) | | | |
| Total solids | 9·00 | 7·78–10·42 | 0·77 | 99 |
| Protein (T.N. × 6·38) | 3·87 | 3·25–4·94 | 0·49 | 117 |
| Lactose (anhydrous) | 4·02 | 3·37–4·43 | 0·34 | 86 |
| Ash | 0·80 | 0·71–0·90 | 0·05 | 105 |
| pH | 6·98 | 6·78–7·41 | 0·18 | — |
| Titratable acidity (ml. 0·1 N-NaOH/100 g. milk) | 9·7 | 3·9–14·9 | 2·8 | 63 |
| | (mg./100 g. milk) | | | |
| Total calcium | 126·1 | 98·3–174·6 | 22·6 | 107 |
| Total magnesium | 12·8 | 10·6–16·6 | 1·5 | 106 |
| Total citric acid | 154 | 108–200 | 27 | 88 |
| Total phosphorus | 84·4 | 68·2–104·8 | 12·0 | 89 |
| Sodium | 110 | 74–152 | 22 | 190 |
| Potassium | 103 | 84–120 | 10 | 74 |
| Chloride | 161·7 | 122·1–215·1 | 23·1 | 155 |
| Casein N | 442·4 | 358·6–553·0 | 58·1 | 110 |
| Lactalbumin + lactoglobulin N | 90·1 | 49·6–116·0 | 18·6 | 137 |
| Proteose–peptone N | 44·3 | 22·2–63·7 | 9·7 | 180 |
| Non-protein N | 29·5 | 15·7–42·2 | 7·9 | 124 |
| Colloidal inorganic Ca | 58·3 | 40·7–91·2 | 15·0 | 117 |
| Caseinate Ca | 36·4 | 24·0–53·1 | 7·7 | 116 |
| Soluble un-ionized Ca | 20·3 | 13·0–29·3 | 4·2 | 80 |
| Ionized Ca | 11·1 | 8·8–14·6 | 1·6 | 97 |
| Colloidal Mg | 5·0 | 4·0–6·2 | 0·6 | 116 |
| Soluble Mg | 7·8 | 6·2–10·4 | 1·0 | 100 |
| Colloidal citric acid | 20 | 9–41 | 9 | 105 |
| Soluble citric acid | 134 | 86–172 | 26 | 85 |
| Colloidal inorganic P | 34·6 | 24·9–53·9 | 8·7 | 118 |
| Casein P | 22·7 | 18·7–28·3 | 2·9 | 106 |
| Soluble inorganic P | 21·2 | 6·6–40·5 | 7·9 | 63 |
| Ester P | 5·8 | 2·0–10·2 | 2·3 | 55 |
| | (mg./g. casein) | | | |
| Caseinate calcium | 12·8 | 10·3–15·1 | 1·7 | 105 |
| Casein phosphorus | 8·1 | 7·2–9·1 | 0·2 | 96 |
| Colloidal inorganic calcium | 20·6 | 14·6–25·8 | 3·7 | 107 |
| Colloidal inorganic phosphorus | 12·1 | 9·0–15·3 | 2·1 | 106 |
| Tricalcium phosphate | 42·2 | 25·9–56·7 | 9·5 | 108 |
| Dicalcium phosphate | 16·0 | 5·3–25·6 | 6·3 | 99 |
| Tri- plus dicalcium phosphate | 58·2 | 42·5–73·5 | 10·3 | 105 |
| Colloidal magnesium | 1·8 | 1·3–2·3 | 0·2 | 106 |
| Colloidal citric acid | 7·0 | 3·1–11·5 | 2·6 | 96 |

### *Chemical composition in relation to stage of lactation*

The relation between the composition of the samples and the stage of lactation of the cows was examined in greater detail by comparing composition and stage of lactation in eighty-five individual cow samples from thirty-six cows, i.e. the samples excluding the herd bulk and subclinical mastitis milks. The relationships of greatest interest are shown in Figs. 1–3.

The concentrations of total nitrogen and of each nitrogen fraction, except proteose-peptone nitrogen, were high at the beginning of lactation but fell rapidly and then increased very gradually (Fig. 1). During the last 10% of lactation, the increases became more rapid so that the concentration of total nitrogen and of the various fractions, including proteose-peptone nitrogen, reached high levels. Except for proteose-peptone

Table 6. *Composition of fat-free milk (seventeen samples) from cows with subclinical mastitis with the mean value for each constituent expressed as a percentage of the corresponding herd bulk mean value*

| | Mean | Range | Standard deviation | Mean as % of herd bulk mean |
|---|---|---|---|---|
| | (g./100 g. milk) | | | |
| Total solids | 8·63 | 8·27–9·20 | 0·93 | 95 |
| Protein (T.N. × 6·38) | 3·27 | 2·69–3·82 | 0·33 | 99 |
| Lactose (anhydrous) | 4·28 | 3·82–4·54 | 0·20 | 91 |
| Ash | 0·77 | 0·73–0·82 | 0·03 | 101 |
| pH | 6·87 | 6·65–7·10 | 0·13 | — |
| Titratable acidity (ml. 0·1 N-NaOH/100 g. milk) | 12·3 | 8·3–14·9 | 1·91 | 80 |
| | (mg./100 g. milk) | | | |
| Total calcium | 115·4 | 103·1–138·4 | 9·2 | 98 |
| Total magnesium | 11·7 | 9·6–14·0 | 1·4 | 97 |
| Total citric acid | 160 | 125–194 | 21 | 91 |
| Total phosphorus | 84·6 | 74·0–94·8 | 5·6 | 89 |
| Sodium | 78 | 59–98 | 12 | 135 |
| Potassium | 138 | 116–160 | 14 | 99 |
| Chloride | 141·0 | 120·5–163·7 | 11·7 | 135 |
| Casein N | 371·8 | 313·0–445·0 | 38·6 | 92 |
| Lactalbumin + lactoglobulin N | 77·6 | 58·8–91·7 | 10·5 | 118 |
| Proteose–peptone N | 38·3 | 21·1–53·9 | 8·4 | 156 |
| Non-protein N | 24·3 | 15·8–33·1 | 4·2 | 102 |
| Colloidal inorganic Ca | 48·7 | 40·4–62·7 | 5·8 | 98 |
| Caseinate Ca | 29·9 | 21·8–38·7 | 4·4 | 95 |
| Soluble un-ionized Ca | 24·1 | 18·5–28·9 | 3·1 | 95 |
| Ionized Ca | 11·6 | 9·7–13·5 | 1·2 | 102 |
| Colloidal Mg | 4·2 | 3·2–5·2 | 0·6 | 98 |
| Soluble Mg | 7·6 | 6·3–9·1 | 1·0 | 97 |
| Colloidal citric acid | 17 | 10–32 | 7 | 90 |
| Soluble citric acid | 143 | 107–182 | 19 | 91 |
| Colloidal inorganic P | 29·6 | 24·2–37·8 | 3·8 | 101 |
| Casein P | 20·3 | 17·3–23·1 | 1·6 | 94 |
| Soluble inorganic P | 27·9 | 20·6–35·4 | 4·1 | 83 |
| Ester P | 6·8 | 4·4–9·6 | 1·5 | 64 |
| | (mg./g. casein) | | | |
| Caseinate calcium | 12·8 | 9·9–15·6 | 1·6 | 105 |
| Casein phosphorus | 8·6 | 7·6–9·3 | 0·5 | 102 |
| Colloidal inorganic calcium | 20·8 | 18·4–24·5 | 1·9 | 108 |
| Colloidal inorganic phosphorus | 12·5 | 11·2–14·8 | 0·9 | 110 |
| Tricalcium phosphate | 40·1 | 29·4–61·8 | 8·5 | 103 |
| Dicalcium phosphate | 19·4 | 4·2–31·5 | 6·6 | 120 |
| Tri- plus dicalcium phosphate | 59·6 | 53·4–70·5 | 4·8 | 108 |
| Colloidal magnesium | 1·8 | 1·3–2·1 | 0·2 | 106 |
| Colloidal citric acid | 7·1 | 4·3–11·9 | 2·4 | 97 |

nitrogen, the concentrations at the end of lactation were much the same as those at the beginning of lactation. Although the concentration of casein was high in early and late lactation, the concentrations of the other nitrogen fractions, especially lactalbumin plus lactoglobulin, were then proportionally even higher with the result that the casein number (casein N/total N × 100) was usually low in these periods.

Fig. 2 illustrates how the concentrations of total calcium and total phosphorus, and of the soluble fractions of these constituents changed with stage of lactation. The concentration of total calcium was generally high both in early and late lactation but in the intervening period no relation with stage of lactation was evident. The levels of the two soluble fractions of calcium were high at the beginning of lactation, but thereafter they

showed little relation to stage of lactation apart from a tendency for the un-ionized fraction to be low at the end of lactation. There was great variation in the concentration of total phosphorus at any particular stage of lactation but there was a general tendency for the values to decrease as lactation advanced. The concentration of soluble inorganic

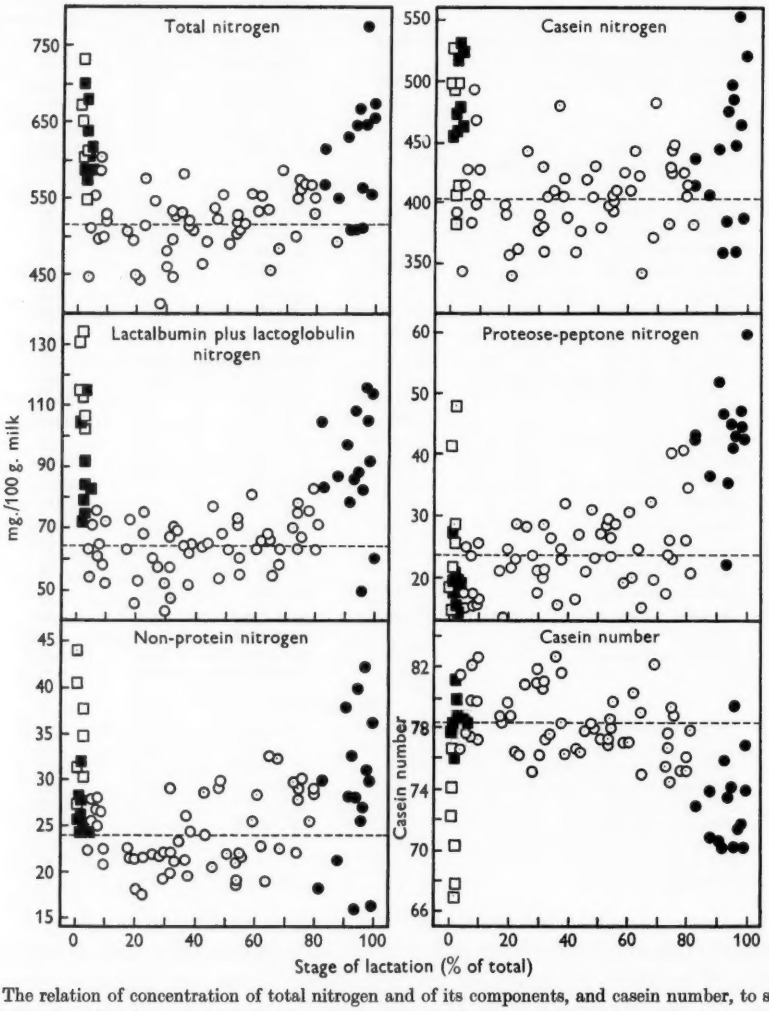


Fig. 1. The relation of concentration of total nitrogen and of its components, and casein number, to stage of lactation in milks from individual cows. (– – –, Mean values for mid-lactation milks; □, colostrum; ■, post-colostrum milk; ○, mid-lactation milk; ●, late lactation milk.)

phosphorus showed no regular change with stage of lactation apart from a marked decrease in late lactation. The amount of ester phosphorus in the samples was closely related to stage of lactation in an inverse curvilinear manner; at the beginning of lactation, the concentration of this phosphorus fraction reached a level about four times the mean value for the mid-lactation samples.

The concentrations of colloidal inorganic calcium and phosphorus, expressed either per 100 g. of milk or per 1 g. of casein, showed much irregular variation during most of the lactation period, but minimum and maximum values occurred in early and late lactation respectively. Variations in caseinate calcium, casein phosphorus, colloidal

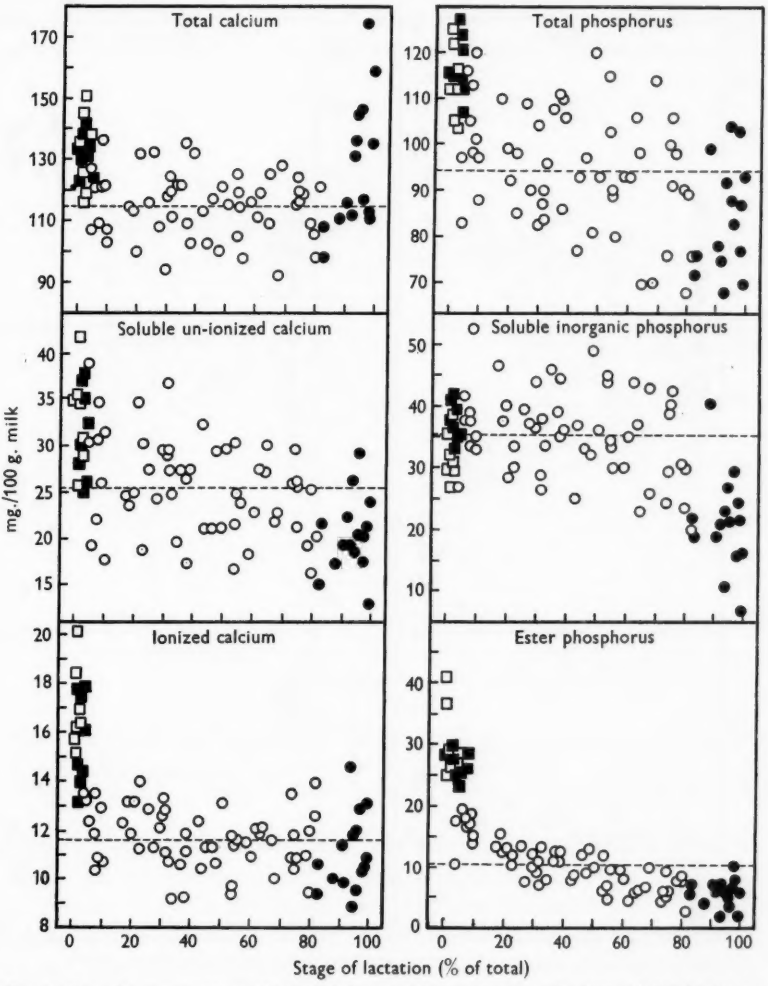


Fig. 2. The relation of concentrations of total calcium, total phosphorus and of the soluble fractions of these constituents to stage of lactation in milks from individual cows. (– – –, Mean values for mid-lactation milks; □, colostrum; ■, post-colostrum milk; ○, mid-lactation milk; ●, late lactation milk.)

magnesium and colloidal citric acid (per g. casein) showed little relation to stage of lactation. The values for total magnesium and citric acid, and for the soluble fractions of these constituents, were also unrelated to stage of lactation.

The variations in values for sodium, chloride, potassium, lactose and acidity with stage of lactation are shown in Fig. 3. The concentrations of sodium and chloride showed

parallel changes; they were both above the mid-lactation average at the beginning of lactation, rapidly decreased to a minimum level, then gradually increased until near the end of lactation when there was a rapid increase to high levels. The changes in lactose concentration during lactation were the opposite of the changes in concentrations of

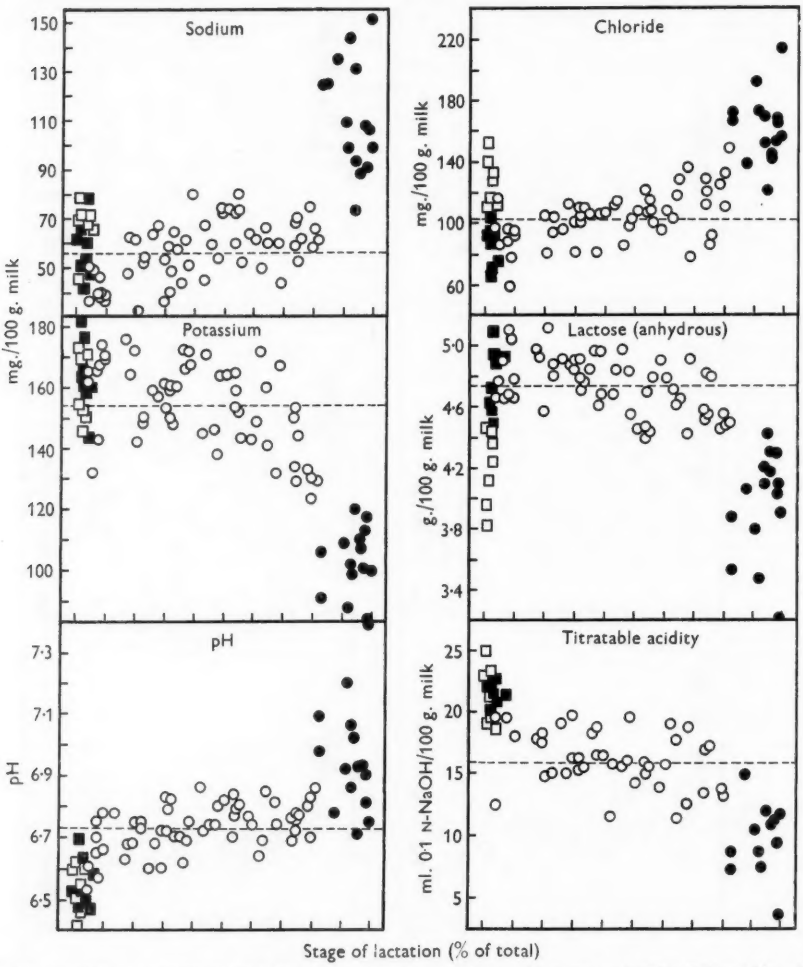


Fig. 3. The relation of concentrations of sodium, chloride, potassium and lactose, and pH and titratable acidity, to stage of lactation in milks from individual cows. (– – –, Mean values for mid-lactation milks; □, colostrum; ■, post-colostrum milk; ○, mid-lactation milk; ●, late lactation milk.)

sodium and chloride, and were similar to the changes in potassium concentration except in the early lactation period when the concentration of potassium tended to be above and not below the mid-lactation average. Fig. 3 shows also that pH was low at the beginning of lactation but increased rapidly to the mid-lactation average value. Thereafter, pH values increased very slowly until near the end of lactation, when the increase became

more rapid. The changes in titratable acidity, as expected, were the reverse of the changes in pH and the very close inverse relation ($r = -0{\cdot}93$, significant at 0·001 level) between these two properties of milk is shown in Fig. 4. This relation has also been observed by Sharp & McInerney (38) who, however, found the inverse relation to be slightly curvilinear.

Results from individual cows whose milk was examined at fairly frequent intervals throughout their lactation confirmed but defined more precisely the changing pattern of composition illustrated in Figs. 1–3. However, these results showed also that with some

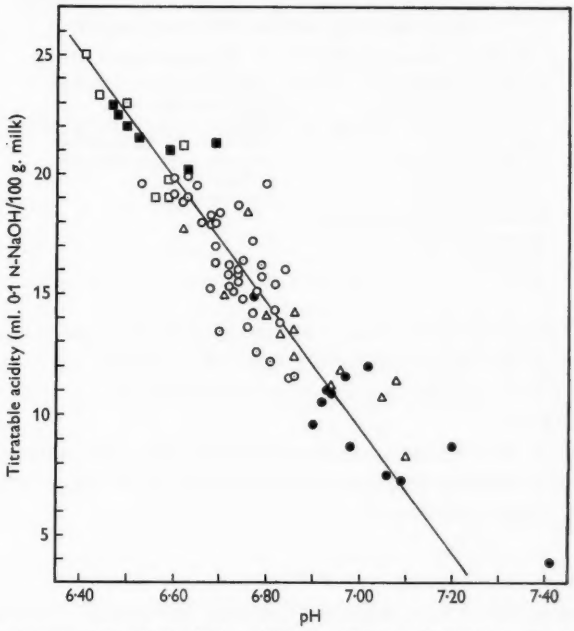


Fig. 4. The relation between pH and titratable acidity in milks from individual cows in different stages of lactation and from cows with subclinical mastitis. (——, calculated regression line; □, colostrum; ■, post-colostrum milk; ○, mid-lactation milk; ●, late lactation milk; △, subclinical mastitis milk.)

cows, the total magnesium content of the milk varied with stage of lactation in the same way as total calcium and that soluble magnesium and soluble citric acid decreased as lactation progressed. With other cows, these relations were not evident. The differences in milk from individual cows probably explains the lack of relation between stage of lactation and the concentrations of total and soluble magnesium and soluble citric acid when the results from all thirty-six cows were considered collectively.

### *Interrelations of milk constituents*

It can be seen from the changes in milk composition with stage of lactation that the concentrations of certain constituents were directly related, e.g. sodium and chloride, and that others were inversely related, e.g. chloride and lactose. The interrelations of the concentrations of the milk constituents were most conveniently seen by plotting each against pH. This was done with the results from all the individual cow samples, except

those from cows with subclinical mastitis. The scatter diagrams revealed the relationships in Table 7. An interesting feature of these relationships was the influence of acidity on the relative amounts of soluble and colloidal calcium and phosphorus in milk. When acidity was high (low pH), the ratio of soluble to colloidal calcium and phosphorus was greater than when acidity was low (high pH). The relationships in Table 7, apart from the one between pH and titratable acidity (in Fig. 4), were not very close.

Table 7. *Relationships between the* pH *of milk and the concentrations of certain milk constituents*

| Inversely related to pH | Directly related to pH |
|---|---|
| Titratable acidity | Colloidal inorganic calcium |
| Total soluble calcium | Caseinate calcium |
| Soluble un-ionized calcium | Colloidal inorganic phosphorus |
| Ionized calcium | Colloidal calcium phosphate |
| Soluble magnesium | Proteose–peptone nitrogen |
| Soluble citric acid | Sodium |
| Total phosphorus | Chloride |
| Soluble inorganic phosphorus | |
| Ester phosphorus | |
| Potassium | |
| Lactose | |

In general, constituents whose concentrations were related to pH in the same way were directly related, e.g. the concentrations of total soluble calcium and ionized calcium, and those related to pH in opposite ways were inversely related, e.g. the concentrations of potassium and sodium.

The relationships between milk composition and stage of lactation, and the interrelations of the milk constituents, were used in Parts II–IV to assist in relating chemical composition and protein stability.

## SUMMARY

1. This paper is Part I of a series dealing with the relation between the chemical composition of milk and the stability of the caseinate complex to ethanol (Part II), rennet (Part III) and heat (Part IV).

2. A description is given of the milk samples, which were taken from herd bulk milk, from individual cows in different stages of lactation and also from cows with subclinical mastitis.

3. The methods used to measure the stability of the caseinate complex to the three coagulating agents are described, together with the methods used to make a detailed chemical analysis of the milk samples.

4. The chemical composition of the samples is given and also the relation between composition and stage of lactation, and the interrelations of the concentrations of certain milk constituents.

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# 713. THE RELATION BETWEEN THE CHEMICAL COMPOSITION OF MILK AND THE STABILITY OF THE CASEINATE COMPLEX

## II. COAGULATION BY ETHANOL

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(With 7 Figures)

### INTRODUCTION

When equal volumes of milk and aqueous ethanol solution are mixed, clots of protein form when the ethanol solution is sufficiently strong. The strength of ethanol necessary to cause coagulation can vary widely with different milks (1). The clots consist mainly of partially denatured casein still associated with calcium and calcium phosphate (2). Since the caseinate dispersion in milk is believed to be colloidally stable by virtue of the hydration and negative charge of the micelles (2), the coagulating effect of ethanol can be attributed to an initial dehydration followed by an aggregation of the destabilized micelles in the presence of calcium and magnesium ions; the bivalency of these cations confers on them a much greater coagulating power towards a negatively charged colloid than the other cations in milk (3). The extent of dehydration and the concentration of bivalent cations necessary for coagulation might be expected to be inversely related and thus, on theoretical grounds, it seems likely that the strength of ethanol required to coagulate an equal volume of milk would be inversely related to the concentration of the bivalent cations.

The most comprehensive study of the coagulation of milk by ethanol is that of Mitamura (1). He examined 2704 milk samples, 927 of which were from Ayrshire cows, and found that the strength of ethanol required to coagulate an equal volume of milk ranged from 66 to 94% with an average of 80% (v/v). In early lactation, milk was very unstable to ethanol but thereafter it gradually became more stable and remained at a fairly constant level of stability that was specific for each cow. Towards the end of lactation, the milk from some cows became more stable to ethanol but a decrease in stability was more common. Relatively unstable milks contained more soluble calcium, soluble magnesium and chloride but less soluble inorganic phosphorus than those more stable to ethanol. Confirmation of the importance of bivalent cations was obtained by adding neutral salts to milk when it was found that the strength of ethanol that caused coagulation decreased as the amount of added bivalent cations increased. Neither pH nor the concentration of fat, colloidal calcium or colloidal phosphorus was related to stability to ethanol.

Sommer & Binney (4) noted that the addition of dipotassium phosphate or sodium citrate to milk, previously made unstable to ethanol by the addition of calcium acetate or magnesium chloride, counteracted the destabilizing influence of the cations. These

results were considered to show the influence of salt-balance on the stability of the caseinate to ethanol. No relation was found between the titratable acidity of fresh milk and its stability to ethanol though developed acidity reduced stability. They observed also that when calcium carbonate was fed to three cows their milk became unstable to ethanol, even though the total calcium content of the milk was unaltered. Eilers (5) confirmed that milk was made less stable to ethanol by the addition of calcium salts or acids, and was made more stable by the addition of bases, and salts whose anions could form weakly dissociated calcium compounds. Seekles & Smeets (6) showed that the 'Utrecht abnormality' of milk, i.e. an instability of the protein to heat and ethanol, was not related to total calcium content and suggested that a high concentration of calcium ions was the cause. This view was supported by the fact that when the concentration of calcium ions in milk was reduced by adding alkali, or anions which could combine with calcium, the stability of the milk to ethanol increased. Seekles & Smeets (6) claimed also that the 'Utrecht abnormality' could be successfully counteracted by supplementary feeding or injection, with sodium citrate.

Further evidence that diet could influence the stability of milk to ethanol was provided by Echenique and Suarez (7,8), who reported that when cows ate plants unusually rich in calcium, their milk was unstable to ethanol though normal in acidity. Also, Hughes & Ellison (9) quoted a report that cows grazing on land with a high calcium content often secreted milk that was unstable to ethanol. Rowlands, Barkworth, Hosking & Kempthorne (10) stated that only one of 618 fresh bulk milks coagulated with 68% ethanol, but with stronger solutions of ethanol the incidence of unstable samples rose sharply, especially in a limestone district.

From the evidence in the literature, there seems little doubt that the concentration of bivalent cations is the most important factor governing the stability of the caseinate complex in milk to ethanol. Because milk contains about 4·5 times as much ionized calcium as ionized magnesium (11), the concentration of calcium ions is probably the most important single factor. The discovery that milk unstable to ethanol contains an abnormally high concentration of calcium ions (12,13,14,15) supports this hypothesis. However, in no investigation has a series of milks differing widely in stability to ethanol been analysed in sufficient detail to enable a relation between stability and chemical composition to be definitely established. This was the object of the present investigation.

## RESULTS

A description of the milk samples, their chemical composition and the way in which their stability to ethanol was determined, has been given in Part I of this series (p. 236).

### *Range of stability to ethanol*

Fig. 1*a* shows that the range of ethanol solutions required to coagulate the twelve herd bulk milks was only 80–84% (v/v) ethanol (average 82% (v/v)). On the other hand, a wide range of stability was found in the milks from individual cows; some were coagulated by the weakest solution used, 66% ethanol, and one required the strongest solution, 90% ethanol. The early lactation milks were very unstable to ethanol (Fig. 1*b*); eleven coagulated with 66–72% ethanol, four with 78–80% ethanol and the over-all average for the group was 70% ethanol. Most of the colostrum samples were coagulated

by 66% ethanol and the rapidity and completeness of coagulation suggested that they would have coagulated with an even weaker solution of ethanol. Fig. 1*c* shows that forty-eight of the fifty-five samples of mid-lactation milk were coagulated by ethanol solutions ranging from 74 to 84%. The average strength of ethanol required for the

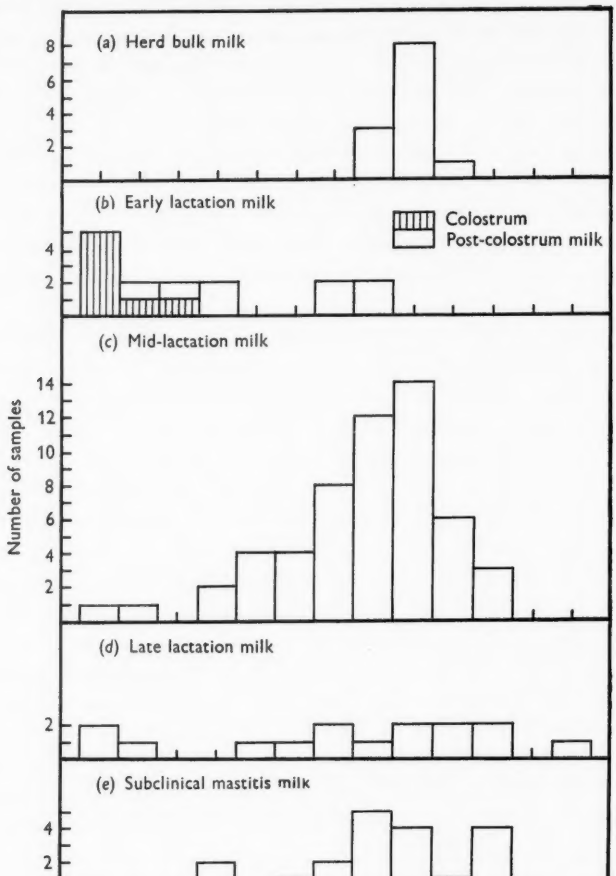


Fig. 1. The distribution of samples in relation to strength of ethanol required for coagulation in each of the five groups of milks.

coagulation of the mid-lactation milks was 80%, a value similar to the average for the herd bulk milks. Both the late lactation and the subclinical mastitis groups of milks had a wide range of stability to ethanol and neither could be classified as stable or unstable (Figs. 1*d*, 1*e*).

The nature of the clots formed by ethanol was variable. Early lactation milk, especially colostrum, gave large, firm clots whereas those formed in late lactation and subclinical mastitis milk were small and easily dispersed. Mid-lactation and herd bulk samples formed clots that were intermediate in size and dispersability.

*Stability to ethanol in relation to stage of lactation*

The way in which stability to ethanol changed during the lactation period was determined by examining the stability of milk from seven cows at intervals during their lactations (Fig. 2). This figure shows that ethanol stability increased rapidly during the first quarter of the lactation period, remained high during the mid-lactation period but that towards the end of lactation the milk from some cows became unstable to ethanol, whereas the milk from others remained stable.

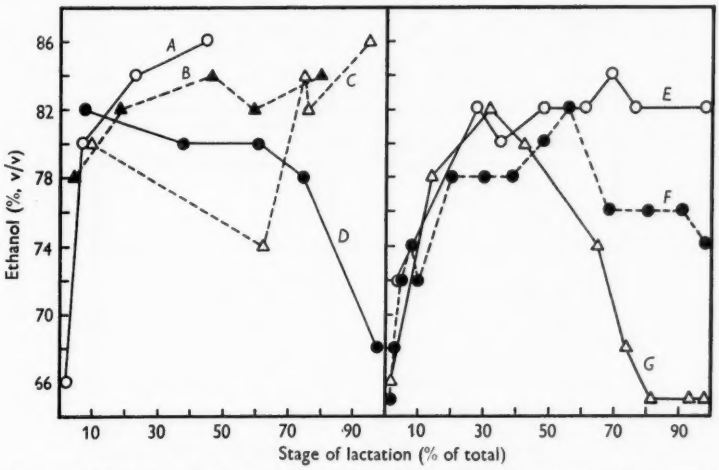


Fig. 2. The relation between stage of lactation and strength of ethanol required for coagulation in milks from seven cows. (Cows *A* and *E* developed subclinical mastitis.)

*Stability to ethanol in relation to chemical composition*

*Herd bulk milk*

The similar stability to ethanol of each of the herd bulk milks (Fig. 1*a*) no doubt resulted from their similarity in chemical composition (Part I, Table 2). These samples differed appreciably only in their sodium, potassium and chloride contents indicating that these constituents did not influence the stability of the caseinate complex. Although the results for the herd bulk milks did not enable the factors controlling ethanol stability to be deduced, they showed that 82% ethanol was the strength required to coagulate milk of average composition.

*Early lactation milk*

The many chemical differences between the early lactation milks and the herd bulk milks have been shown in Part I, Table 3. The higher concentrations of ionized calcium and soluble magnesium, and possibly the lower pH values of the early lactation milks seemed the most likely causes of their lower stability to ethanol.

When the chemical composition and stability of the individual early lactation milks were compared, only a few relationships were detectable (Fig. 3). Stability tended to decrease as the concentration of ionized calcium increased, and to increase as pH increased. The concentrations of non-protein nitrogen and chloride appeared to be inversely

related to ethanol stability, but these relations were probably only consequences of the parallel changes in the concentrations of these two constituents and of ionized calcium during the early lactation period (Part I, Figs. 1–3).

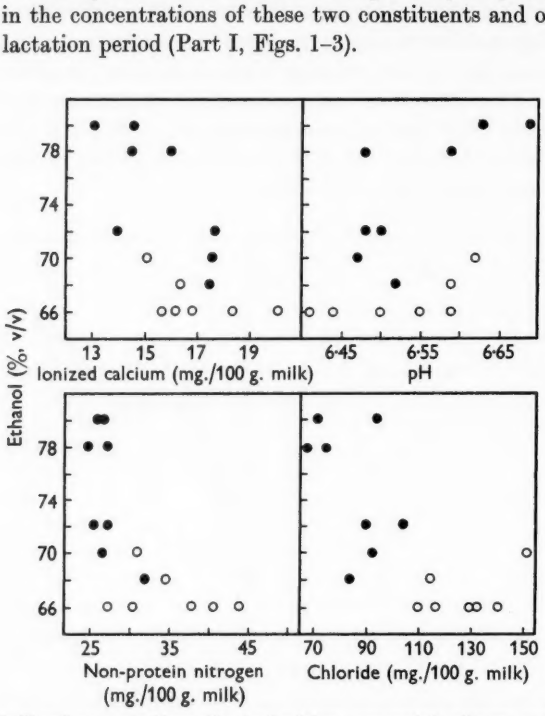


Fig. 3. The relation of pH and concentrations of ionized calcium, non-protein nitrogen and chloride to strength of ethanol required for coagulation in early lactation milks. (○, Colostrum; ●, post-colostrum milk.)

*Mid-lactation milk*

As this group contained milks of widely differing ethanol stability and chemical composition (Fig. 1c; Part I, Table 4), it provided greater possibility of relating stability to composition than the two groups already examined. Chemical composition and ethanol stability were first compared by separating the samples into subgroups, each containing milks coagulated by the same strength of ethanol, and calculating the mean chemical composition of the milks in each subgroup. The results showed that as stability to ethanol increased, the mean concentrations of ionized calcium, soluble un-ionized calcium and soluble citric acid decreased, and that the mean pH values and the mean concentrations of colloidal calcium phosphate and lactalbumin plus lactoglobulin increased.

A comparison of the chemical composition and stability of the individual mid-lactation milks confirmed the above relationships and revealed also a weak inverse relation between the concentration of soluble magnesium and stability. Scatter diagrams illustrating four of these somewhat weak relationships are shown in Fig. 4. The concentration of ionized calcium was more closely related to ethanol stability than any of the other compositional factors; the correlation coefficient was −0·64 (significant at 0·001 level) which indicated that the variation in the concentration of this constituent accounted for about 40% of the variation in the stability to ethanol of the mid-lactation samples. From

statistical analyses kindly made by Dr D. J. Finney, F.R.S., it appeared that the other relationships were due mainly to the correlation of the levels of the particular constituents with that of ionized calcium in the samples. The results in Fig. 4 for the milks coagulated by 66 and 68% ethanol respectively provided further evidence that the concentration of ionized calcium was more important in controlling ethanol stability than the concentration of the other milk constituents determined.

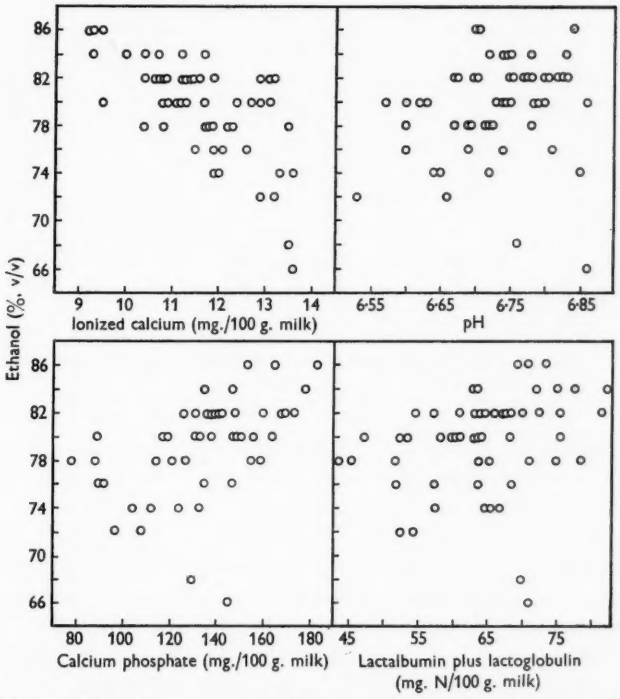


Fig. 4. The relation of pH and concentrations of ionized calcium, calcium phosphate and lactalbumin plus lactoglobulin nitrogen to strength of ethanol required for coagulation in mid-lactation milks.

*Late lactation milk*

The late lactation milks were generally richer in sodium, chloride and non-casein nitrogen, and poorer in lactose, potassium, soluble inorganic phosphorus and ester phosphorus than the herd bulk milks (Part I, Table 5), yet they were neither markedly stable nor unstable to ethanol (Fig. 1*d*). It appeared, therefore, that the stability of the caseinate complex was independent of the concentrations of all these constituents. The high pH values of the late lactation milks likewise conferred no bias towards a stability consistently lesser or greater than that of the herd bulk milks. The fact that ionized calcium and soluble magnesium were the only constituents whose concentrations in the late lactation milks were very variable and yet had practically the same mean values as in the herd bulk milks, suggested that the levels of these two constituents were responsible for the type of distribution of ethanol stability shown in Fig. 1*d*.

When the concentrations of the chemical constituents of the individual late lactation milks were plotted against the corresponding concentrations of ethanol required for coagulation, only the relationships shown in Fig. 5 were found. As in the early and mid-lactation groups, the concentration of ionized calcium was inversely related to ethanol stability. The direct relation seen here between the concentration of soluble inorganic phosphorus and ethanol stability was not detectable in the other groups of milks. The concentration of soluble magnesium, and pH, were not related to ethanol stability in the late lactation samples.

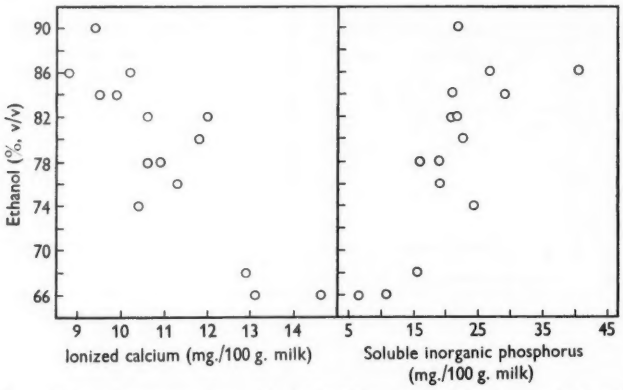


Fig. 5. The relation of concentrations of ionized calcium and soluble inorganic phosphorus to strength of ethanol required for coagulation in late lactation milks.

*Subclinical mastitis milk*

The subclinical mastitis milks were somewhat similar to the late lactation milks both in the distribution of their stability to ethanol (Fig. 1*e*) and in their differences in chemical composition when compared with the herd bulk milks (Part I, Table 6). Therefore, as with the late lactation milks, it appeared that the stabilities of the subclinical mastitis milks were determined by the variation in the concentrations of constituents whose mean concentrations for the group were not abnormal, e.g. ionized calcium and soluble magnesium.

When the chemical composition and stability of the individual subclinical mastitis milks were compared, the closest relationships found were those in Fig. 6. The concentrations of ionized calcium, total magnesium and soluble magnesium were inversely related to ethanol stability, whereas pH was directly related. The latter relation, as in the early and mid-lactation groups, could be attributed to the inverse relation between pH and the concentration of ionized calcium in milk (Part I, Table 7).

*All milks from individual cows*

It has been shown that an inverse relation between the concentration of ionized calcium and stability to ethanol existed in each of the four groups of milks from individual cows. When the values for these two variables in all the individual cow samples were compared and the regression line calculated, it was found that most samples fitted the line reasonably well (Fig. 7). The correlation coefficient was −0·76 (significant at 0·001

level) which indicated that about 60% of the variation in stability to ethanol could be attributed to the variation in the concentration of ionized calcium. The slope of the regression line showed that an increase in the calcium ion content of milk of 1 mg./100 g. would, on average, decrease the strength of ethanol causing coagulation by 4% (v/v). The line indicated also that a milk containing 11·3 mg. of ionized calcium per 100 g. would be coagulated by 82% (v/v) ethanol, a relation that agreed well with the average results for the herd bulk milks (11·4 mg. of ionized calcium per 100 g. and 82% ethanol required for coagulation).

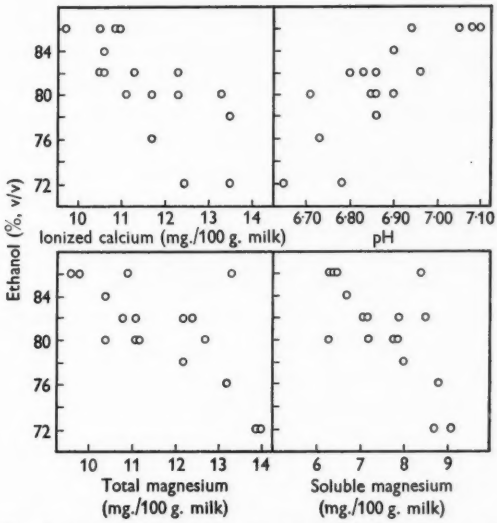


Fig. 6. The relation of pH and concentrations of ionized calcium, total magnesium and soluble magnesium to strength of ethanol required for coagulation in milks from cows with subclinical mastitis.

When the results for all samples were examined collectively, no other relation between chemical composition and stability to ethanol was found apart from those already detected in the individual groups of milks and which could probably be attributed to the interrelation of the concentration of ionized calcium and the concentrations of the milk constituents in question.

The applicability of the salt-balance theory to the data was tested also. Sommer & Hart (16) suggested that the salt-balance in milk, i.e. the ratio of total calcium plus magnesium to total phosphate plus citrate in gram-equivalents, was related to the stability of the caseinate complex to heat. In 1923, Sommer & Binney (4) used this concept to explain the variation in stability to ethanol of different milks. In the present instance, it seemed more reasonable to calculate a salt-balance in terms of the soluble fractions of the above four constituents since all the evidence indicated that the composition of the aqueous phase controlled the stability to ethanol of the colloidal caseinate complex. This was done for each milk by adding together the milliequivalent amounts (per 100 g. milk) of soluble calcium and magnesium and subtracting from this total the combined milliequivalent amounts of soluble phosphate and citric acid. For this purpose, the soluble phosphate was partitioned into $H_2PO_4^-$ and $HPO_4^{2-}$ by means of Sorensen's

phosphate-buffer table (17) to enable the concentration of each fraction to be calculated. The calculation showed that the concentration of the anions always exceeded the concentration of the cations and that the greater the excess, the greater the stability of the milk to ethanol, a result in agreement with the general theory of salt-balance. However, since

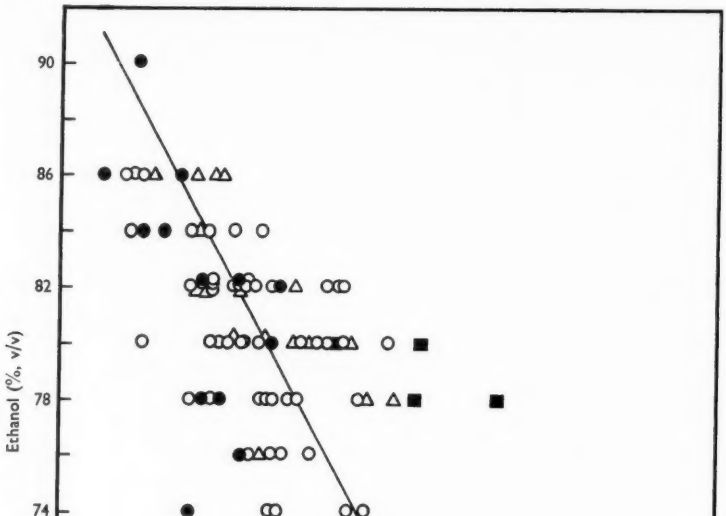


Fig. 7. The relation between concentration of ionized calcium and strength of ethanol required for coagulation in milks from individual cows in different stages of lactation and from cows with subclinical mastitis. (——, Calculated regression line; □, colostrum; ■, post-colostrum milk; ○, mid-lactation milk; ●, late lactation milk; △, subclinical mastitis milk.)

there was an inverse relation between the excess of anions over cations and the concentration of ionized calcium, the relation between salt-balance and stability to ethanol could be simply and reasonably attributed to the interrelation of salt-balance and concentration of ionized calcium.

## DISCUSSION

The range of ethanol solutions required to coagulate the caseinate complex in 132 samples of milk from Ayrshire cows was very similar to that found by Mitamura (1) for 2704 milks from cows of several breeds; the average strength of ethanol causing coagulation was

identical in both experiments, namely, 80% (v/v) ethanol. Both investigations showed also that early lactation milk, especially colostrum, was very unstable to ethanol, that as lactation advanced milk became more stable within fairly wide limits, and that at the end of lactation stability varied markedly from cow to cow. Insufficient late lactation milks were examined in the present investigation to confirm the report of Mitamura that stability to ethanol usually decreased in late lactation.

The prediction was confirmed that the greater the concentration of ionized calcium in milk the less stable would be the caseinate complex to ethanol. This relation applied to milk from cows in early, mid- or late lactation and also to milk from cows with subclinical mastitis. When the results for all the milks from individual cows were considered, about 60% of the variation in stability to ethanol could be accounted for by the variation in the concentration of ionized calcium. This fact explained the instability to ethanol of the colostrum and post-colostrum samples whose average contents of ionized calcium were respectively 17·0 and 15·6 mg./100 g. of milk, whereas the corresponding values for the herd bulk milks, mid- and late lactation and subclinical mastitis milks, all with little bias towards low or high stability, were 11·4, 11·6, 11·1 and 11·6 mg./100 g. of milk respectively. The manner in which the concentration of ionized calcium in milk changed with stage of lactation (Part I, Fig. 2) thus explained the lactational changes in the ethanol stability of milk described above.

Although it appeared that some other factor in addition to calcium ion concentration influenced the stability of the caseinate complex to ethanol, especially in the post-colostrum milks, the results did not indicate what this factor might be. In some of the groups of milk, pH and the concentrations of certain constituents, e.g. chloride, non-protein nitrogen, soluble un-ionized calcium, soluble magnesium and soluble inorganic phosphorus were related to ethanol stability but in every instance the relationship could be attributed to an interrelation of the concentration of the constituent with that of ionized calcium in the particular group. Some of these relationships between composition and stability were noted also by Mitamura (1). No doubt the ionized fraction of soluble magnesium would augment the coagulating power of the calcium ions but the lack of a suitable method for determining magnesium ions prevented this opinion from being tested. Neither the concentration of the caseinate-phosphate complex nor such aspects of its chemical composition as were determined appeared to be related to stability to ethanol. The low concentration of the caseinate complex in the subclinical mastitis milks seemed a possible explanation of the looseness of the clots formed by ethanol in this type of milk but the formation of similar clots in late lactation milk, which was comparatively rich in caseinate, made this proposal untenable.

The salt-balance theory of Sommer & Binney (4) appeared to be unnecessary in explaining the variation in the stability to ethanol of the caseinate complex in milk. This variation could be explained more directly and in accordance with the theories of colloid stability, mainly in terms of the concentration of calcium ions. Although relatively high concentrations of ionized calcium were found only in early lactation milk, this investigation confirmed the view of Smeets, Seekles and Boogaerdt (12, 13, 14, 15) that when marked instability to ethanol is encountered the principal cause is an abnormally high concentration of calcium ions in the milk.

SUMMARY

1. The variation in the stability of milk protein to ethanol and the relationship between milk composition and ethanol stability were examined.

2. Samples of herd bulk milk were very similar in stability to ethanol; the range of aqueous ethanol solutions required to coagulate the caseinate complex in an equal volume of milk was only 80–84% (v/v) ethanol. Samples from individual cows showed a wide variation in stability; coagulation was caused by ethanol solutions ranging in strength from 66 to 90% (v/v) ethanol.

3. Colostrum was very unstable to ethanol but stability rapidly increased during the post-colostrum period to higher levels in mid-lactation. Late lactation and subclinical mastitis milk showed no definite bias towards stability or instability.

4. The strength of ethanol required to coagulate the caseinate complex in an equal volume of milk was inversely related to the concentration of ionized calcium in the milk. The correlation coefficient was $-0{\cdot}76$ (significant at 0·001 level) indicating that approximately 60% of the variation in stability was accounted for by the variation in the concentration of ionized calcium.

5. The relationships between the concentrations of other milk constituents and stability to ethanol could be attributed to the interrelations of the concentrations of these constituents and the concentration of ionized calcium.

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# 714. THE RELATION BETWEEN THE CHEMICAL COMPOSITION OF MILK AND THE STABILITY OF THE CASEINATE COMPLEX

## III. COAGULATION BY RENNET

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(With 7 Figures)

### INTRODUCTION

It has been known for many years that the coagulation of milk by rennet occurs in two stages. Recent investigations have shown that the essential reaction in the first or enzymic stage is the rapid liberation of non-protein nitrogen from α-caseinate with the formation of α-paracaseinate (1,2,3,4,5,6). The temperature coefficient ($Q_{10° C.}$) of this reaction at a pH typical of milk, 6·7, was found to be about 1·9 between 1 and 30° C., and at constant rennet concentration and constant temperature the rate of the reaction increased as pH decreased from 6·7 to 5·5 (6). According to Pyne (7), the rate of the first stage of coagulation increases as the concentration of ionized calcium increases. It has been reported also that the hydration and charge of the caseinate micelles are reduced during the first stage (8,9).

In the second or non-enzymic stage, the α-paracaseinate, together with the unchanged β- and γ-caseinates coagulates provided calcium ions or other alkaline earth ions are present and the temperature is within the range 15 to about 45° C. The temperature coefficient of the second stage of coagulation is large, namely, 1·3–1·6 per 1° C. (10). The magnitude of this temperature coefficient led Berridge (10) to suggest that the second-stage reaction is a heat denaturation of the paracaseinate micelles followed by their aggregation, with calcium ions acting as 'bridges'. Normally, all the α-caseinate is converted to α-paracaseinate before coagulation occurs (1,2), but when the concentration of calcium ions is sufficiently large, a premature precipitation of incompletely formed paracaseinate can take place (11). The rate of the second stage of coagulation is appreciably increased by only a small increase in the concentration of calcium ions (11,12). Pyne (11) states that pH has little influence on the second stage but according to Smith & Bradley (13), a decrease in pH accelerates the second stage as well as the first.

Much of the above information on the coagulation of milk by rennet was obtained by studying each stage separately. Of more practical interest are the attempts that have been made to relate the over-all time taken by both the enzymic and non-enzymic reactions to form a coagulum, i.e. the renneting time of milk, to the chemical composition of the milk.

No close or consistent relation has been established between renneting time and the concentration of total or soluble calcium in milk. There is evidence that milks with long renneting times tend to be low in calcium content (14,15,16,17), but Ling (18) found that the

renneting time of milk increased as the soluble calcium content increased, and he suggested that salt-balance might influence renneting time. On the other hand, it is well known that when calcium salts are added to milk renneting time decreases (10,13,19,20,21,22,23), and that when some calcium is inactivated or removed, renneting time increases (22,24). When about 20% of the calcium is removed, coagulation does not occur (24,25). The addition of magnesium ions is also known to decrease renneting time (22,23). Berridge (26) states that the accelerated coagulation in the presence of added bivalent cations is due partly to the decrease they cause in the pH of milk but with calcium ions, it is known that the accelerating effect persists even when pH is kept constant. There is the possibility that renneting time may depend to some extent on the concentration of trace metals in milk since the addition of small amounts of some heavy metal salts (e.g. Mn, Cd, Zn) shortens renneting time and the addition of others (e.g. Co, Cu, Ni) lengthens it (23,27).

It is well known that the acidity of milk is an important factor governing renneting time, although there appears to be a lack of direct experimental evidence showing how the natural variation in the acidity of milk from individual cows is related to the variation in renneting time. Some evidence of a tendency for renneting time to increase as acidity decreases has been provided by Webb, Holm & Deysher (14) and Ling (18,34), and Sanders, Matheson & Burkey (28) have given average values for groups of milks from individual cows showing that there was a progressive increase in renneting time as pH increased from 6·32–6·39 to 6·80–7·45; the longest average renneting time was approximately nine times the shortest. There is ample indirect evidence of an inverse relation between acidity and renneting time, e.g. the addition of acid to milk or the natural souring of milk shortens renneting time (19,20,22,34). Presumptive evidence of an inverse relation between acidity and renneting time is the increase in renneting time with advancing lactation (pH increasing) (20,29,30) and the long renneting times common with subclinical mastitis milk (pH often high) (28,31,32,33).

There are conflicting views on whether differences in the caseinate complex are partly responsible for the variation in the renneting time of milks. It would appear that the greater the calcium phosphate content of the caseinate complex the larger are the complex micelles (35,36,37) and the shorter is renneting time (38,39). The possibility of an inverse relation between renneting time and the particle size of the caseinate complex is also suggested by reports that when milk is homogenized the size of the caseinate micelles increases (39,40) and renneting time decreases (40,41,42). Chevalier, Mocquot, Alais and Bonnat (16,17,43) believe that the physical and chemical constitution of the caseinate complex, as well as the composition of the aqueous phase, determines the renneting time of milk. On the other hand, Hostettler, Rychener & Künzle (35) found no difference in the calcium and phosphorus contents of corresponding caseinate fractions obtained by centrifuging normal and slow renneting milks, and Pyne (7) states that the composition of the aqueous phase, and not that of the caseinate complex, determines renneting time.

According to Browne (44), the greater the concentration of colloidal calcium phosphate in milk, the firmer is the coagulum formed by rennet. However, it is generally agreed that the firmness of the coagulum depends mainly on the concentration of the caseinate complex, a low concentration resulting in a loose curd and a high concentration resulting in a firm curd (28,33,45,46,47). These investigations indicate also that the concentration of the caseinate complex in milk is unimportant in relation to renneting time except with soft-curd milk which is occasionally obtained from disease-free cows. This type of milk

has a normal pH but is invariably deficient in the caseinate complex and with rennet, slowly forms a soft curd.

Much information is now available on the factors which regulate each stage of coagulation, and also on the effect of added substances on the renneting time of milk, but the relation between the chemical composition of raw milk and renneting time has not been adequately examined. The lack, until recently, of a suitable method for estimating calcium ions has made it impossible to determine the influence of the concentration of this milk constituent on renneting time and only average data are presented in the most extensive comparison of pH and renneting time so far reported(28). The object of the present investigation was to attempt to relate renneting time and milk composition by making a detailed chemical analysis of a series of milks with a wide range of renneting times.

## RESULTS

A description of the milk samples, their chemical composition and the way in which renneting time was determined have been given in Part I of this series (p. 236).

### *Range of renneting times*

A wide variation was found in the renneting times of the milks examined; the range was 1·4–12·9 min. and two samples did not coagulate. There was also much variation in the firmness of the coagula. The average renneting time of the twelve herd bulk milks was 4·1 min. with a range of 3·2–5·4 min. (Fig. 1*a*). All the herd bulk milks gave a firm curd. Most of the samples comprising the two groups of early lactation milk, colostrum and post-colostrum, were rapidly coagulated (Fig. 1*b*) and formed a very firm curd. The average renneting time of the seven colostrum samples was 2·1 min. (range 1·4–3·7 min.) and that of the eight post-colostrum samples was 2·2 min. (range 1·4–3·0 min.). The renneting times of the fifty mid-lactation samples (Fig. 1*c*) ranged from 2·0 to 6·4 min. with an average of 3·7 min., a value similar to the herd bulk milk average. Eight of the nine mid-lactation milks that took longer than 5 min. to coagulate came from cows whose udders had been injected with dihydrostreptomycin or aureomycin a few days before sampling because of injury to the udder or because the milk had slightly high bacterial and cell contents. There was no detectable abnormality in the chemical composition of these milks. The coagula formed by the mid-lactation milks were in general similar in firmness to those of the herd bulk milks. Two of the fifteen late lactation milks did not coagulate, and the renneting times of the remainder ranged from 4·2 to 12·9 min. with an average of 7·9 min. (Fig. 1*d*). Most of the late lactation milks formed a very soft curd. The renneting times of the sixteen milks from cows with subclinical mastitis ranged from 2·3 to 11·8 min. (Fig. 1*e*). This wide range was probably, to some extent, a reflexion of a variation in the severity of infection. The subclinical mastitis milks with a renneting time greater than 4·5 min. formed a very soft curd, whereas those coagulating more quickly gave the normal, firm curd typical of the herd bulk and mid-lactation milks.

### *Renneting time in relation to stage of lactation*

A comparison of Fig. 1*b–d* indicates that renneting time tended to increase as lactation progressed and this was confirmed by comparing the renneting times of all the samples from individual cows, excluding subclinical mastitis milks, with the corresponding stages

of lactation (Fig. 2). This figure shows that there was a rapid initial increase in renneting time followed by a slow, gradual increase until near the end of lactation when the increase became much greater. The eight samples indicated in Fig. 2, and whose renneting times appeared abnormally long for the stage in lactation of the cows, were those obtained from the cows that had been treated with antibiotic (see above).

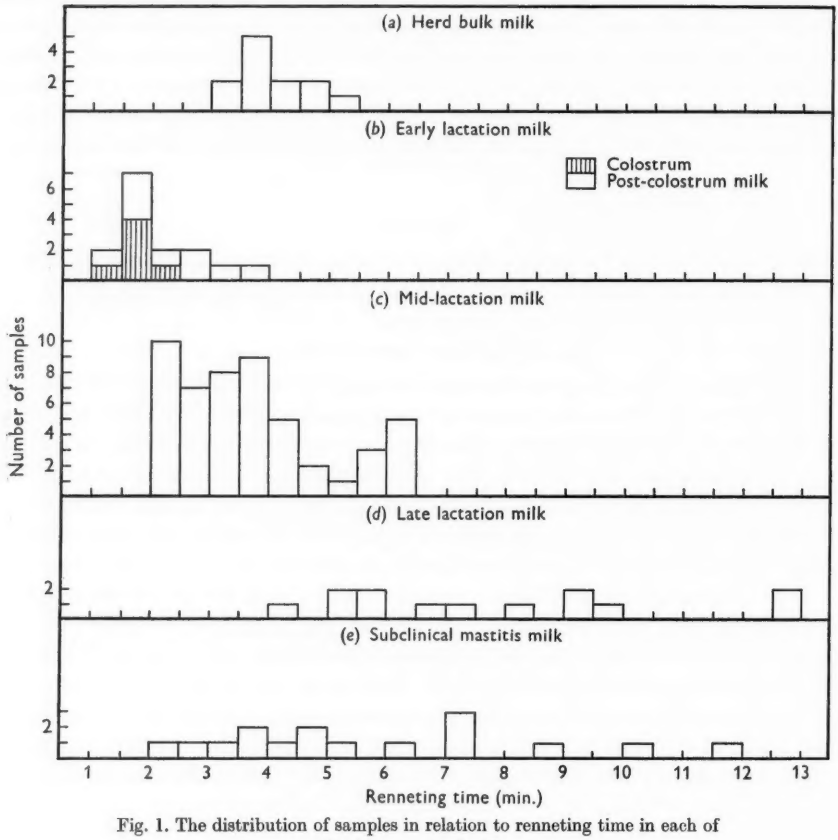


Fig. 1. The distribution of samples in relation to renneting time in each of the five groups of milks.

The determination of the renneting time of milks from each of seven cows sampled at intervals confirmed that renneting time increased as lactation progressed. This relation between renneting time and stage of lactation was detectable even in the herd bulk milks whose renneting time increased from a minimum of 3·2 min. in November to a maximum of 5·4 min. in August, months in which the average stage of lactation of the herd was respectively at a minimum of 88 days and at a maximum of 206 days.

### *Renneting time in relation to chemical composition*

#### *Herd bulk milk*

The closest relationships found between chemical composition and renneting time are shown in Fig. 3; an increase in renneting time was associated with an increase in pH and

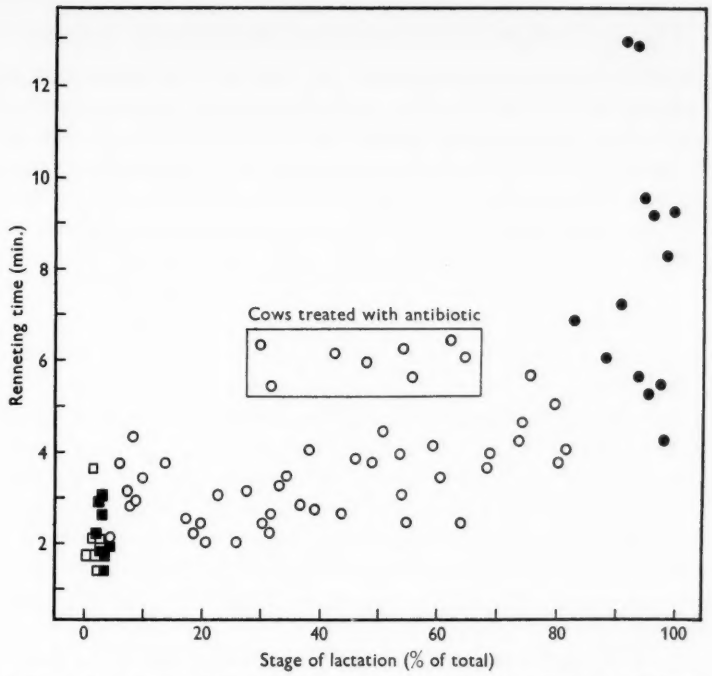


Fig. 2. The relation between stage of lactation and renneting time in milk from individual cows. (□, Colostrum; ■, post-colostrum milk; ○, mid-lactation milk; ●, late lactation milk.)

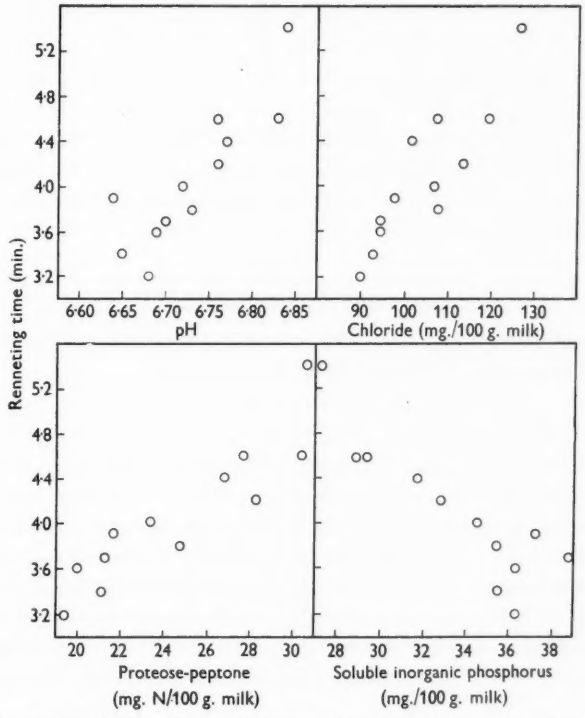


Fig. 3. The relation of pH and concentrations of chloride, proteose–peptone nitrogen and soluble inorganic phosphorus to renneting time in herd bulk milks.

in the concentrations of chloride and proteose-peptone, and with a decrease in the concentration of soluble inorganic phosphorus. In addition to these relations, values for titratable acidity, lactose, total and ester phosphorus, total soluble magnesium and soluble un-ionized calcium tended to be inversely related to renneting time, whereas with lactalbumin plus lactoglobulin, non-protein nitrogen and sodium the reverse relation tended to be true. There was no relation between the concentration of ionized calcium and renneting time.

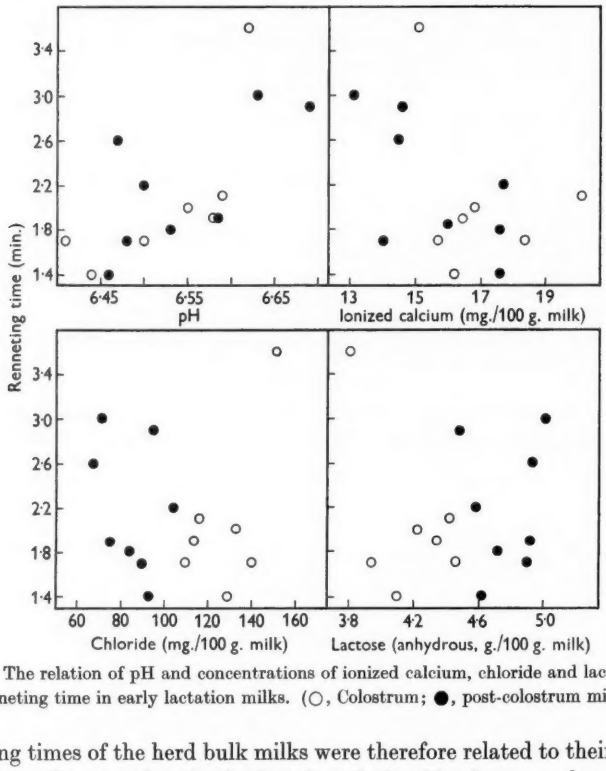


Fig. 4. The relation of pH and concentrations of ionized calcium, chloride and lactose to renneting time in early lactation milks. (○, Colostrum; ●, post-colostrum milk.)

The renneting times of the herd bulk milks were therefore related to their pH values in the expected way. It seemed probable that the relationships between the concentrations of other milk constituents and renneting time could be attributed to the interrelation of these concentrations and pH (Part I, Table 7).

*Early lactation milk*

An examination of the chemical composition of the early lactation milks (Part I, Table 3) suggested that the rapidity of their coagulation was caused by their low pH and high content of ionized calcium. The firmness of their coagula could possibly be attributed to a high concentration of the caseinate complex. The association reported in the literature between a decreased calcium phosphate content of the caseinate complex and long renneting times and soft coagula did not hold with these early lactation milks.

There was little close relation between composition and renneting time within the early lactation group of milks. However, as pH increased renneting time also increased and

there was a tendency for an increase in calcium ion concentration to be associated with a decrease in renneting time (Fig. 4). In addition, the concentration of chloride tended to be inversely related, and that of lactose to be directly related to renneting time, but these relationships could have resulted from the interrelation of the levels of these two constituents and pH in early lactation (Part I, Fig. 3). The fact that the relation between chloride concentration and renneting time in the early lactation group of milks was the opposite of that in the herd bulk milks suggested that this constituent had no direct influence on renneting time.

*Mid-lactation milk*

In comparing the chemical composition and renneting time of the mid-lactation milks, the eight samples from cows treated with antibiotic a few days before sampling were omitted. Most of the remaining samples coagulated within the narrow range of 2·0–4·5 min. Although there was appreciable variation in the chemical composition of the mid-lactation milks (Part I, Table 4), a comparison of the chemical composition and renneting time of the individual samples did not reveal any close relationships. Renneting time tended to be directly related to pH and to the concentration of lactalbumin plus lactoglobulin, and inversely related to the concentrations of potassium, soluble unionized calcium and soluble magnesium. Neither the concentration of ionized calcium nor the composition of the caseinate complex was related to renneting time.

*Late lactation milk*

Of the many differences in chemical composition between the herd bulk milks and the late lactation milks (Part I, Table 5), the considerably higher pH values of the latter appeared the most likely cause of their longer renneting times. The average concentration of ionized calcium in the late lactation milks was only slightly less than that in the herd bulk milks but some of the late lactation milks had the lowest contents of ionized calcium encountered. As in the early lactation groups of milks, the relation between the calcium phosphate content of the caseinate complex and both renneting time and coagulum firmness were the opposite of what might be expected. Another apparent anomaly was that the late lactation milks formed soft coagula despite their average content of caseinate complex being 10% greater than that of the herd bulk milks.

The relationships between chemical composition and renneting time in this group of milks were again not very close (Fig. 5), but as pH increased and the concentration of ionized calcium decreased, renneting time increased. There was also some indication that renneting time was inversely related to the concentration of soluble magnesium and directly related to the concentration of sodium (Fig. 5). The latter relation could have resulted from the parallel changes in the levels of pH and sodium in late lactation (Part I, Fig. 3).

The data for samples A and B in Fig. 5 indicate that renneting time was much more closely linked to pH than to the concentration of ionized calcium. Sample A took 12·8 min. to coagulate and sample B did not coagulate yet each contained more ionized calcium than any of the other late lactation milks. Both the samples had high pH values, 7·06 and 7·41 respectively, which were consistent with the general relation between pH and renneting time shown by the other late lactation milks.

*Subclinical mastitis milk*

Although the subclinical mastitis milks had a wide range of renneting times, 2·3–11·8 min., most had a renneting time exceeding the average renneting time of the herd bulk milks, 4·1 min. The chemical composition of the subclinical mastitis milks (Part I, Table 6) indicated that the cause of this tendency towards slow coagulation might be high pH values as in the late lactation group. The average concentration of ionized calcium

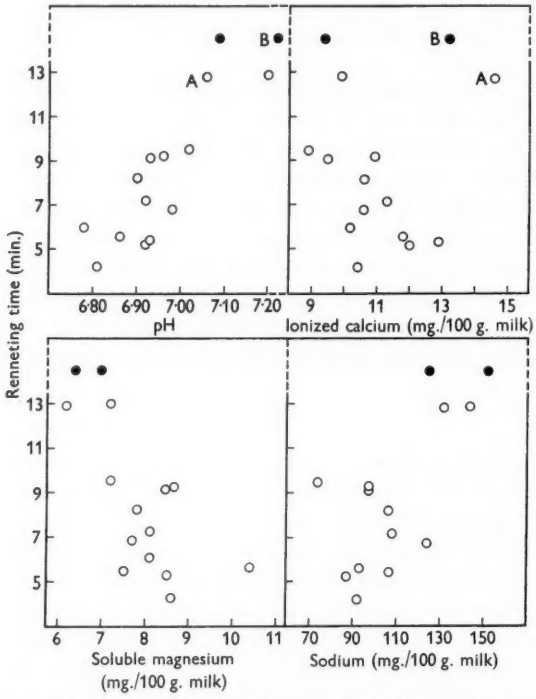


Fig. 5. The relation of pH and concentrations of ionized calcium, soluble magnesium and sodium to renneting time in late lactation milks. (●, Samples that did not coagulate.) as shown; samples A and B, see p. 273).

was almost the same as in the herd bulk milks. The softness of the coagula from the subclinical mastitis milks slow to coagulate was certainly not caused by a deficiency of calcium phosphate in the caseinate complex but might have been caused by a low concentration of the complex.

The best within-group relationships between chemical composition and renneting time are shown in Fig. 6. As in the other groups of milks, renneting time increased as pH increased. The tendency noted in the early and late lactation milks for renneting time to decrease as the concentration of ionized calcium increased was also detectable in this group of milks. There were indications of inverse relations between renneting time and the concentrations of soluble un-ionized calcium, and potassium. In addition, more rapid coagulation was usually associated with higher concentrations of total calcium, total phosphorus, soluble inorganic phosphorus, soluble citrate and lactose, and with a lower

concentration of chloride. Most of these relationships, however, could probably be attributed to the interrelation of the concentrations of the constituents and pH (Part I, Table 7). It appeared also that there was an inverse relation between casein number and renneting time but this relationship, noted also in some of the other groups of milks, was unlikely to be of significance since as far as is known the serum proteins take no part in the rennet coagulation of raw milk.

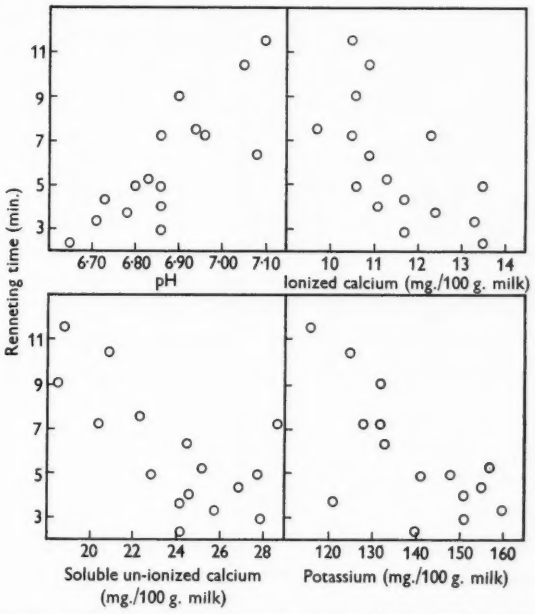


Fig. 6. The relation of pH and concentrations of ionized calcium, soluble un-ionized calcium and potassium to renneting time in milk from individual cows with subclinical mastitis.

*All milks from individual cows*

In each of the groups of milks from individual cows, the variation in the concentrations of the milk constituents that appeared important in relation to renneting time was small compared with the variation over all the samples. To compare renneting time with composition over the whole range of compositional values, the data for all milks from individual cows were examined collectively. This procedure gave the relationships shown in Fig. 7. As pH increased from 6·4 to 7·2, there was a curvilinear increase in renneting time from 1·5 to 13 min. Above pH 6·8, the increase in renneting time with increasing pH was greater than between pH 6·4 and 6·8. The association between acidity and renneting time was also shown by the inverse curvilinear relation between titratable acidity and renneting time (Fig. 7).

Although high concentrations of ionized calcium were invariably associated with short renneting times, when the concentration of this constituent was less than about 12 mg./100 g. of milk, it bore little relation to renneting time (Fig. 7). There were also weak inverse relationships between renneting time and the concentrations of soluble

inorganic phosphorus (Fig. 7), soluble un-ionized calcium, soluble magnesium, ester phosphorus, potassium and lactose. On the other hand, renneting time tended to increase as the concentrations of sodium, chloride and proteose-peptone increased. The

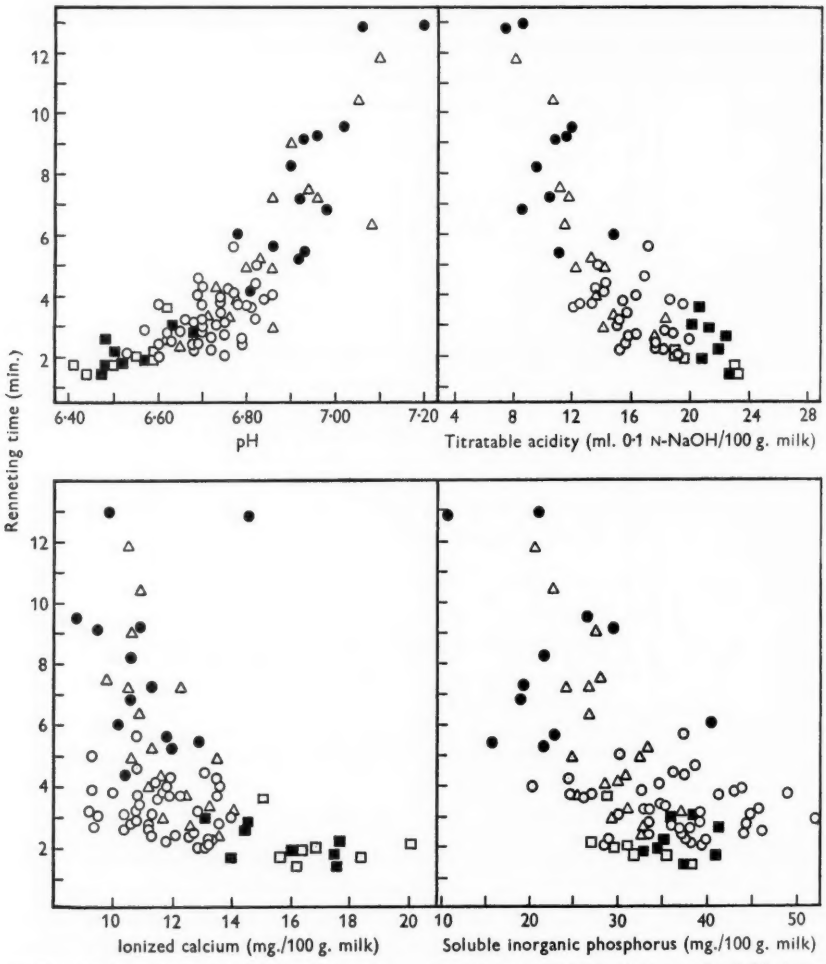


Fig. 7. The relation of pH, titratable acidity and concentrations of ionized calcium and soluble inorganic phosphorus to renneting time in milks from individual cows in different stages of lactation and from cows with subclinical mastitis. (□, Colostrum; ■, post-colostrum milk; ○, mid-lactation milk; ●, late lactation milk; △, subclinical mastitis milk.)

existence of all these weak relationships could probably be attributed to the interrelation of the concentrations of the milk constituents and pH (Part I, Table 7). Renneting time was not related to the composition or the concentration of the caseinate complex. Neither was there any relation between renneting time and salt-balance, which was calculated as described in Part II, p. 263.

All the above relationships were confirmed when a comparison was made of composition

and renneting time in series of milks taken from six cows at different times. In each of the six series of samples, the direct relation between renneting time and pH was close and a particular pH value was associated with much the same renneting time.

## DISCUSSION

In all the groups of milk examined, namely herd bulk, early lactation, mid-lactation, late lactation and subclinical mastitis, it was evident that as pH increased so did renneting time. Within each group, the relation appeared linear but when all the samples from individual cows were considered, the general relationship was seen to be curvilinear (Fig. 7). This relation is similar to that found by Sanders *et al.* (28), but they noted a marked decrease in the rate of coagulation once the pH exceeded 6·65. Although renneting time was more closely related to pH than to any other property of the milks, there was appreciable variation in renneting time at any given level of pH, especially in the mid-lactation milks. The reason for this variation, noted also by Peltola (48), was not evident from the available data; neither variation in the concentration of ionized calcium nor in the calcium phosphate content of the caseinate complex appeared to be responsible.

The titratable acidity of the milks was almost as good a guide to renneting time as pH. The inverse curvilinear relation between titratable acidity and renneting time (Fig. 7) was expected in view of the close inverse relation found between titratable acidity and pH (Part I, Fig. 4).

The reason for the importance of pH in relation to renneting time is suggested by the work of Nitschmann & Böhren (6). They have shown that when temperature and rennet concentration are constant, the rate of conversion of casein to paracasein decreases as pH increases from 5·5 to 6·7. Presumably the rate of conversion will continue to decrease with further increases in pH until in the region of pH 7–7·5 rennet activity ceases. Since it appears that the conversion of caseinate to paracaseinate is complete or nearly so before the typical coagulation of milk occurs (1, 2, 11), it follows that renneting time will increase as pH increases. The rapid coagulation of the early lactation milks, especially colostrum, and the slow coagulation of the late lactation milks and some of the subclinical mastitis milks, could therefore be attributed to the respectively low and high pH values of these milks. When milk does not coagulate with rennet the most likely cause will be an abnormally high pH value, e.g. the two late lactation milks that failed to coagulate had pH values of 7·09 and 7·41 respectively.

The reports of McDowall, Dolby & McDowell (20), Mattick & Hallett (29) and Konovalov (30) that the renneting time of milk increases with the stage of lactation of the cow were confirmed in this investigation (Fig. 2). The reason for this relation is probably the dependence of renneting time on pH; the changes in the renneting time of milk during the lactation period followed the same pattern as the changes in pH (cf. Fig. 2; Part I, Fig. 3).

Although the milks containing a relatively high concentration of ionized calcium coagulated quickly, those with an average or below average content of this constituent (12 mg. or less per 100 g. milk) had very variable renneting times which were unrelated to their calcium ion content (Fig. 7). The weak relationship between calcium ion concentration and renneting time suggested that at the temperature at which the renneting time of the samples was determined (30° C.), the variation in the rate of the non-enzymic reaction was small in relation to the variation, due to pH differences, in the rate of the

enzymic reaction. The dominant influence of pH on renneting time was illustrated in samples that had high pH values and very long renneting times despite a content of ionized calcium above average (samples A and B, Fig. 5). However, the existence of a general inverse relation between pH and the concentration of ionized calcium in milk (Part I, Table 7) meant that when conditions were conducive to a rapid first-stage reaction, i.e. low pH, they were also generally conducive to a rapid second-stage reaction, i.e. high calcium ion concentration, and vice versa.

When the results from all the individual cow samples were considered it was found that renneting time increased as the concentration of sodium, chloride and proteose-peptone increased and the concentration of soluble inorganic phosphorus, soluble un-ionized calcium, soluble magnesium, ester phosphorus, potassium and lactose decreased. None of these relationships was close and each of them could probably be attributed to the interrelation of pH and the level of these constituents in the milks (Part I, Table 7). But there was the possibility that some of these constituents might have a specific effect on rennet action. Chloride ions appear to inhibit rennet action (3,23,49,50), and magnesium ions influence the non-enzymic reaction in the same way as calcium ions (22,23). However, it seems unlikely that the weak general relationships between renneting time and the concentrations of chloride and soluble magnesium were of any great significance; the relation between chloride concentration and renneting time in the early lactation group of milks was the opposite of that in the other groups, and magnesium ions are only half as effective as calcium ions in promoting the non-enzymic reaction (11) and their molar concentration in milk is only about one-third that of calcium ions (51,52).

There was no relation between salt-balance and renneting time, a result in agreement with the findings of Holm, Webb & Deysher (14) but at variance with those of Ling (18). None of the colloidal mineral constituents of milk appeared to have any influence on renneting time. The concentration of calcium phosphate, whether expressed as mg. per 100 g. of milk or per 1 g. of casein, was not related to renneting time. This supports the view of Pyne (7) that the natural variation in the calcium phosphate content of the caseinate complex is insufficient to affect renneting time. However, there is the possibility that variations in the composition of the caseinate complex other than those determined in this investigation may partly determine renneting time and thereby be a reason for the variation in renneting time of milks with the same pH. Chevalier *et al.* (16,17,43) believe that physical and chemical differences in the caseinate complex, e.g. in the ratio of Ca to N, particle size, affinity for water and relative amounts of $\alpha$-, $\beta$- and $\gamma$-casein, as well as differences in the composition of the aqueous phase, are responsible for variations in the renneting time of milk.

The present investigation confirmed the generally held view that the concentration of caseinate in milk is unrelated to renneting time. It was true that the early lactation milks, in which the average concentration of caseinate was high, coagulated rapidly and that the subclinical mastitis milks, in which the average caseinate level was low, tended to be slow to coagulate but the late lactation milks were also slow to coagulate despite their average caseinate content being almost as high as that of the colostrum samples. In contrast to findings in other investigations (28,33,45,46,47), the concentration of caseinate in the milks was not consistently related to the firmness of the coagula. Early lactation milk, rich in caseinate, formed a firm curd and subclinical mastitis milk, when deficient in caseinate, formed a loose curd, but late lactation milk, which contained almost as much caseinate

as colostrum, also gave a loose curd. The reason for this apparent anomaly may have been the incomplete formation and coagulation of paracaseinate as a result of the high pH values and the relatively low concentrations of ionized calcium common in late lactation milk.

SUMMARY

1. The variation in the time required for the coagulation of milk by rennet and the relationship between milk composition and renneting time were examined.

2. The renneting times of herd bulk milks varied over the relatively narrow range of 3·2–5·4 min., whereas the renneting times of milks from individual cows varied from 1·4 to 12·9 min. and two did not coagulate.

3. In general, milk quick to coagulate gave a firm curd (early lactation milk) and milk slow to coagulate gave a soft curd (late lactation and subclinical mastitis milk).

4. The renneting time of milk was related to the stage of lactation of the cow. In early lactation, renneting times were short and as lactation advanced there was a progressive increase in renneting time; the increase became more pronounced towards the end of lactation.

5. The property of milk most closely related to renneting time was acidity. As pH increased from 6·4 to 7·2, there was a curvilinear increase in renneting time from 1·5 to 13 min. Titratable acidity was related to renneting time in the opposite way to pH.

6. Although milk containing a relatively high concentration of ionized calcium coagulated quickly with rennet, the renneting time of milks containing average, or less than average, amounts of ionized calcium was not related to their content of this constituent.

7. Weak relationships between the concentrations of other milk constituents and renneting time could be attributed to the interrelations of the concentrations of these constituents and pH.

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# 715. THE RELATION BETWEEN THE CHEMICAL COMPOSITION OF MILK AND THE STABILITY OF THE CASEINATE COMPLEX

## IV. COAGULATION BY HEAT

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(With 5 Figures)

### INTRODUCTION

When milk of normal composition is heated at 160° C., the caseinate complex coagulates in a few minutes (1); as the temperature is lowered, the time required for coagulation increases logarithmically until at temperatures approaching 100° C. several hours elapse before coagulation ensues (1,2). The time required at a given temperature for coagulation to occur can vary widely with different milks (1,3,4,5). Attempts have been made to relate the heat stability and chemical composition of milk but with limited success.

Although the effect of heat on the lactalbumin and lactoglobulin in milk may include aggregation (6,7,8,9) and possibly some complex formation with caseinate (10,11,12), in addition to denaturation (13,14,15,16), there is no evidence that the serum protein content of normal milk is related to the heat stability of the caseinate complex. In fact, Pyne & McHenry (5) found that milk from which most of the lactalbumin and lactoglobulin had been removed coagulated in exactly the same time at 120° C. as the unaltered milk. Nevertheless, the high concentration of serum proteins in colostrum may well be responsible for its marked instability to heat. Colostrum is especially rich in lactoglobulin (17) whose components, the immunizing globulins, are more rapidly denatured by heat than any of the other serum proteins (18,19). There is apparently a critical concentration of lactoglobulin above which the heat-denatured protein either flocculates and causes a sympathetic coagulation of the caseinate micelles or aggregates on the caseinate micelles to such an extent that they are unable to play their normal protective role and coagulate. According to Engel & Schlag (20), the critical concentration is 1 %, or according to Davies (21) it is 0·9% (including lactalbumin), and is exceeded in milk for about 3 days after calving.

For many years, the variation in the heat stability of the caseinate complex in milk other than colostrum has been attributed to differences in the mineral composition of the milk, largely as a result of the salt-balance theory of Sommer & Hart (3,22,23). According to this theory, there is an optimum value for the ratio of total calcium plus magnesium to total phosphate plus citrate in milk and any deviation from this value, occurring naturally or by the addition of salts, results in a decrease in the heat stability of the caseinate complex. Sommer & Hart stated that an excess of the bases was the most common cause of instability. The usefulness of the salt-balance theory can be criticized on the grounds that the theory was established mainly by the effect of added salts on the heat stability of milk, that the so-called optimum ratio can apparently vary from milk to milk and that difficulty has been found in relating the experimentally determined

salt-balance to heat stability (4). Whatever truth there may be in this theory, a practical drawback appears to be that existing analytical methods are insufficiently accurate to enable the heat stability of milk to be predicted from the salt-balance.

It has been suggested that when the total or ionized calcium content of milk is abnormally high, the milk will be unstable to heat (24,25,26,27). This suggestion was supported by the investigations of Smeets, Seekles and Boogaerdt (28,29,30,31,32) who found that when the calcium ion content of milk ultrafiltrate exceeded about 15 mg./100 ml., the milk was unstable to heat; the normal range was 8–14·4 mg./100 ml. In addition, Pyne & McHenry (5) reported an inverse, curvilinear relation between the 'effective' calcium ion concentration in milk (measured by a renneting technique) and coagulation time at 130° C. They suggested also that the greater the calcium phosphate content of the caseinate complex, the less stable was the complex to heat. Pyne & McHenry concluded that if the concentration of calcium ions and the calcium phosphate content of the caseinate complex were high, only a moderate degree of developed acidity and caseinate denaturation would cause coagulation, which would then be fairly rapid. If, on the other hand, the levels of the two predetermining factors were low, a greater development of acidity and more denaturation would be necessary before coagulation ensued; under these conditions, coagulation would be relatively slow. Further evidence of the possible importance of calcium in relation to heat stability has been obtained by its addition to, or removal from, milk. For example, the addition of soluble calcium salts to milk reduces heat stability (2,25,33,34), whereas the inactivation of some calcium by the addition of citrate or phosphate, increases heat stability (2,33,35). Webb & Holm (2) noted, however, that when the concentration of added phosphate or citrate exceeded a certain level, there was an abrupt decline in heat stability.

There does not appear to be any relation between the natural acidity of milk and the stability to heat of the caseinate complex (3,4,23), but when acid is added to milk, stability is usually lessened (2,36,37). Miller & Sommer (37) found that the decrease in heat stability of acidified milk was most marked between pH 6·4 and 6·2. Suggested reasons for the decreased heat stability of the caseinate complex in acidified milk are a decrease in the negative charge of the micelles with increased aggregation (38) or a change in the ratio of $H_2PO_4^-$ to $HPO_4^{2-}$ (23). Changes in the equilibrium between the colloidal and soluble mineral constituents of milk may also be responsible. For example, a decrease in the pH of milk causes an increase in the concentration of soluble calcium and phosphorus at the expense of the calcium and calcium phosphate content of the caseinate complex (39,40,41,42,43). The citric acid associated with the caseinate complex also passes into the aqueous phase when milk is acidified (44).

It is important to bear in mind that the heat coagulation of the caseinate complex is preceded by many complex chemical changes in the milk whose significance in relation to the mechanism of coagulation is by no means fully understood. In addition to the denaturation of the serum proteins, the casein molecule itself is appreciably altered. Howat & Wright (45,46) showed that when calcium caseinate solutions were heated at temperatures ranging from 90 to 120° C. there was a rapid liberation of phosphorus as well as of some nitrogen. They noted that visible coagulation occurred when about 45% of the phosphorus had been liberated from the caseinate, and since dephosphorylation and coagulation both had a temperature coefficient ($Q_{10°\,C.}$) of about 3, it was concluded that these reactions were related, a view supported by Torboli (33). They noted also that solutions of

casein and dephosphorylated casein containing more than sufficient calcium to combine with the casein were less heat stable than when just sufficient calcium was present. Another heat-induced reaction is one between casein and lactose (47,48). It is detectable at temperatures below 100° C. (49) and is mainly responsible for the brown colour of more severely heated milk (50). It is not known whether this reaction is normally involved in the coagulation mechanism, but Pyne & McHenry (5) have shown that the caseinate complex in a lactose-free milk can be coagulated by heat, though more slowly than when lactose is present. The accelerating effect of lactose may be due simply to acidity derived from its decomposition when heated (5,51,52,53) and possibly from its linkage with the basic amino-groups of the caseinate (49). It is well established that when milk is heated the concentrations of soluble calcium and phosphorus decrease (39,54,55,56,57,58,59) and recently the concentration of ionized calcium has also been shown to decrease (60).

The inference from many of the investigations on heat stability is that the concentration of calcium ions in milk is the most important single factor determining the stability of the caseinate complex. Although this theory is attractive in view of the likelihood of an inverse relation between the zeta-potential of the negatively charged caseinate particles and the concentration of bivalent cations, the direct evidence in its favour is by no means conclusive. Seekles and his colleagues (28,29,30,31,32) did not establish the existence of a relation between the concentration of ionized calcium and heat stability in milk with a wide range of coagulation times but only that when the calcium ion content exceeded a certain level, the milk was likely to be unstable to heat. And Pyne & McHenry's results (5) showed that over limited ranges of coagulation time, the relation between heat stability and calcium ion concentration was not close. In no experiment as yet has a comparison been made of the detailed chemical composition of milks varying widely in stability to heat. This was the purpose of the present investigation, and by utilizing one of the recently developed chemical methods of estimating ionized calcium, it was hoped to establish whether the concentration of this or of some other constituent in milk was related to the stability to heat of the caseinate complex.

## RESULTS

A description of the milk samples, their chemical composition and the method used to determine their coagulation times at 130, 140 and 150° C. have been given in Part I of this series (p. 236).

Table 1 shows the mean and range of the coagulation times at the three temperatures for each group of milks, and also the mean and range of the values for the temperature coefficient of the coagulation reaction. Coagulation at 140° C. was, on average, 2·0–3·7 times more rapid than at 130° C., and at 150° C. it was 1·5–4·1 times more rapid than at 140° C. When the logarithm of coagulation time was plotted against temperature, an approximate straight-line relation was found for most samples. Samples that did not show a linear relation were those whose exact point of coagulation was difficult to detect and those that coagulated before the bath temperature was reached. Examples of the former were confined to late lactation and subclinical mastitis milks and examples of the latter were confined to colostrum samples. Because of the general proportionality of the coagulation times at the three temperatures, only the coagulation times at 130° C. were used as a measure of the relative heat stabilities of the samples. These values were

preferred because at 130° C. the coagulation times were longer, and hence the differences between samples larger and the percentage error smaller, than at the two higher temperatures. In addition, a temperature of 130° C. is closer to the temperatures to which milk is subjected in some commercial processes.

Table 1. *The mean and range of the coagulation times at* 130, 140 *and* 150° C. *of groups of milks (fat-free) together with the mean and range of values for the temperature coefficient of the coagulation reaction between* 130 *and* 150° C.

| Milk group (no. of samples) | Coagulation time (min.) Mean 130° C. | Mean 140° C. | Mean 150° C. | Range 130° C. | Range 140° C. | Range 150° C. | Temperature coefficient ($Q_{10°\,C.}$) Mean 130–140° C. | Mean 140–150° C. | Range 130–140° C. | Range 140–150° C. |
|---|---|---|---|---|---|---|---|---|---|---|
| Herd bulk (12) | 29·9 | 12·3 | 3·2 | 17·2–59·0 | 6·8–26·2 | 2·6–5·1 | 2·4 | 3·4 | 1·9–2·9 | 2·6–5·3 |
| Colostrum (7) | 2·6 | 1·1 | 0·7 | 0·6– 8·6 | 0·6– 2·4 | 0·5–1·2 | 2·0 | 1·5 | 1·1–3·5 | 1·1–2·0 |
| Post-colostrum (8) | 23·0 | 9·8 | 4·0 | 16·2–31·8 | 6·9–13·9 | 2·7–5·6 | 2·3 | 2·5 | 2·3–2·4 | 2·1–3·7 |
| Mid-lactation (55) | 28·1 | 10·6 | 3·2 | 10·6–70·3 | 3·5–28·9 | 1·7–7·2 | 2·8 | 3·2 | 1·9–3·6 | 2·1–5·4 |
| Late lactation (15) | 45·4 | 14·7 | 3·4 | 15·3–86·2 | 3·2–35·0 | 1·4–5·6 | 3·7 | 4·0 | 1·6–11·2 | 2·3–6·2 |
| Subclinical mastitis (17) | 29·4 | 10·4 | 2·4 | 8·0–64·2 | 2·6–18·2 | 1·1–3·6 | 3·0 | 4·1 | 1·3–8·6 | 1·8–6·3 |

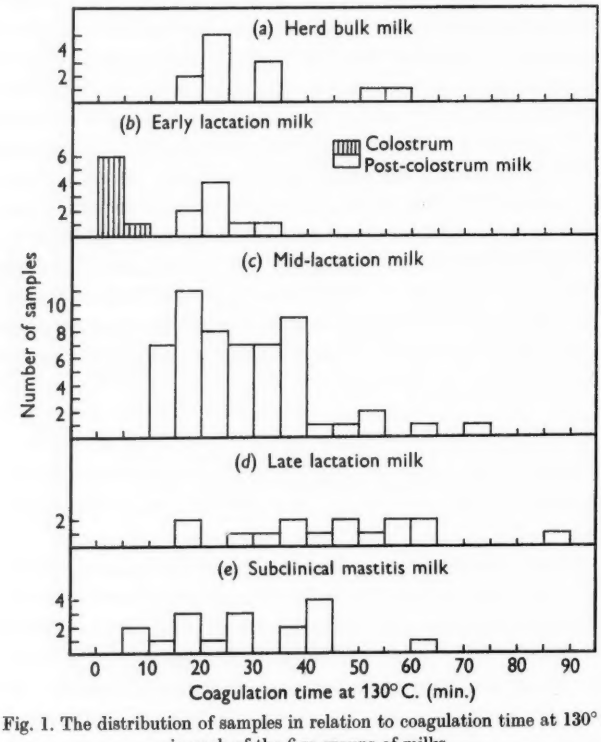
(a) Herd bulk milk
(b) Early lactation milk
Colostrum
Post-colostrum milk
(c) Mid-lactation milk
(d) Late lactation milk
(e) Subclinical mastitis milk
Number of samples
0 10 20 30 40 50 60 70 80 90
Coagulation time at 130° C. (min.)

Fig. 1. The distribution of samples in relation to coagulation time at 130° C. in each of the five groups of milks.

### *Range of coagulation times*

The coagulation times of the milk samples at 130° C. had a wide range, namely 0·6–86·2 min. The range of coagulation times in each group of milks is given in Table 1, and the distribution of coagulation times in Fig. 1. Ten of the twelve herd bulk milks had

coagulation times ranging from 17·2 to 33·9 min. but the remaining two were much more stable and took 52·5 and 59·0 min. respectively to coagulate. There was some evidence (see below) that this variation, which appeared seasonal, was related to the changes in the average stage of lactation of the herd. The two groups of early lactation milks differed greatly in stability to heat. The seven colostrum samples were very unstable and, with the exception of one whose coagulation time was 8·6 min., all coagulated in the range 0·6–2·6 min.; the mean coagulation time of these six samples was only 1·6 min. The eight post-colostrum samples were similar in stability to the herd bulk samples (Fig. 1*b*) and had a mean coagulation time of 23·0 min. Most of the fifty-five mid-lactation samples coagulated within 10–40 min.; only six took longer than 40 min. (Fig. 1*c*). As would be expected, the average coagulation time of the mid-lactation samples (28·1 min.) was almost the same as that of the herd bulk milks (29·9 min.). Since nine of the fifteen late lactation milks took longer than 40 min. to coagulate, it appeared that this type of milk had a tendency to be more stable to heat than mid-lactation milk (Fig. 1*d*). It was difficult to detect the exact point of coagulation of the more stable late lactation milks because the clots which formed were very small and their detection made difficult by the deposit which formed on the tube during the prolonged heating. The seventeen subclinical mastitis milks had much the same range of coagulation times as the mid-lactation milks (Fig. 1*e*). It was difficult to detect the point of coagulation of some of the subclinical mastitis samples for the same reasons as with certain of the late lactation milks.

*Coagulation time in relation to stage of lactation*

The distribution of the coagulation times in Fig. 1 suggested that heat stability increased with stage of lactation. This trend was detectable also in the herd bulk milks whose coagulation times tended to increase as the average stage of lactation of the herd increased from about 100 days in the winter months to about 200 days in late summer (see Part I, Table 1). However, when a comparison was made of the coagulation times of the samples from individual cows, excluding subclinical mastitis milks, and the stage of lactation of the cows, the relation was not close. All that could be said was that in the first 5% of the lactation period there was a rapid increase in coagulation time as colostrum was succeeded by milk less abnormal in composition and that there was a tendency for the late lactation milks (from the last 10% of the lactation period) to have the longest coagulation times; in the intervening wide 'mid'-lactation period, there was no relation between coagulation time and stage of lactation.

Coagulation time and stage of lactation were also compared by examining the milk of eight cows at intervals during their lactations. The results confirmed the general conclusions already drawn and emphasized that the variation in coagulation time with stage of lactation was usually erratic and differed from cow to cow. Similar observations have been made by Holm, Webb & Deysher (4).

*Coagulation time in relation to chemical composition*

*Herd bulk milk*

Although the herd bulk milks varied considerably in stability to heat there was little close relationship between their chemical composition and coagulation times. High stability appeared to be associated with high pH values (low titratable acidity) and there were weak inverse relationships between coagulation time and the concentrations of total

calcium, total phosphorus and soluble inorganic phosphorus (Fig. 2). There was no relation between stability and the concentration of casein or of the serum proteins. However, it appeared that when the concentration of casein relative to the other proteins was small, i.e. when the ratio of lactalbumin plus lactoglobulin to casein was large and the

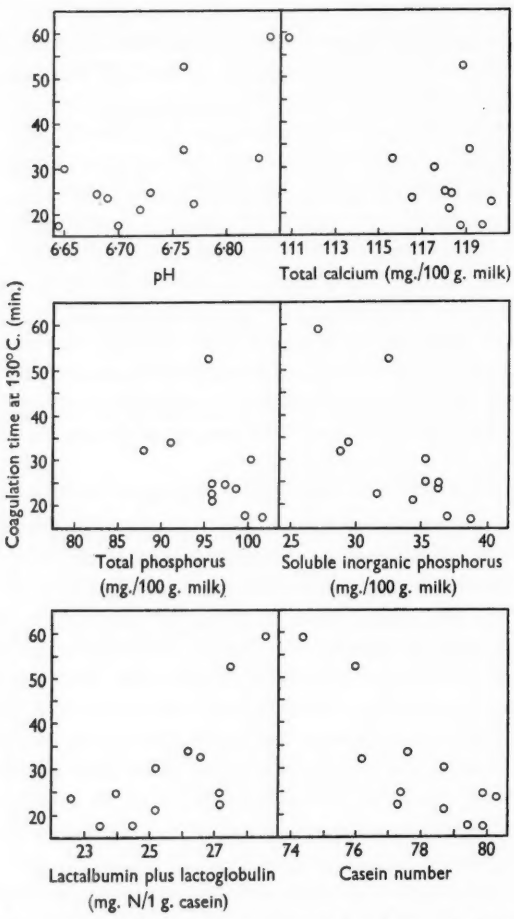


Fig. 2. The relation of pH, concentrations of total calcium, total phosphorus, soluble inorganic phosphorus and lactalbumin plus lactoglobulin nitrogen, and casein number, to coagulation time at 130° C. in herd bulk milks.

casein number was small, the samples were more stable to heat (Fig. 2). As this relation was the opposite of what might reasonably be expected, it was probably coincidental. The same could also be said of weak direct relationships between coagulation time and the concentrations of sodium, chloride and non-protein nitrogen and inverse relations between coagulation time and the concentrations of potassium and lactose. The association of lower stability with higher concentrations of lactose may, however, have been significant since lactose is the main source of developed acidity in heated milk (5).

There was no relation between coagulation time and either the concentration of calcium ions or the composition of the caseinate complex, both of which varied little in the herd bulk milks (Part I, Table 2).

*Early lactation milk*

Reasons for the differences in stability between the two groups of early lactation milk were suggested from a comparison of the average chemical composition of these two types of milk with that of the herd bulk milks (Part I, Table 3). The unstable colostrum samples contained, on average, about 2·5 times as much lactalbumin plus lactoglobulin as the herd bulk milks. The colostrum samples were also rich in ionized calcium; their average content was 17·0 mg./100 g. of milk, whereas the herd bulk milk average was only 11·4 mg./100 g. The concentration of soluble magnesium, and presumably also of magnesium ions, was also appreciably higher in the colostrum samples than in the herd bulk milk samples. The low pH values of the colostrum samples might have predisposed towards instability to heat. There were other marked differences in composition between the colostrum and herd bulk milk samples, e.g. in ester phosphorus, non-protein nitrogen, soluble un-ionized calcium and lactose contents, but it seemed unlikely that these could be responsible for the different average stability to heat of these groups of milks. Although the post-colostrum milks differed in composition from the herd bulk milks in the same respects as the colostrum samples, the differences were smaller and did not result in the post-colostrum milks being less stable to heat than the herd bulk milks. The fact that the post-colostrum milks contained, on average, even more caseinate than the colostrum samples indicated that a high caseinate concentration *per se* did not cause instability to heat.

In view of the similarity of the coagulation times of the colostrum samples, it was not surprising that no relation between composition and stability was detectable within this group of samples. Although there was greater variation in the stability of the post-colostrum milks, the only relationships between composition and stability were an increase in coagulation time as pH increased and as the concentration of calcium ions decreased. Neither relation was close.

*Mid-lactation milk*

The average chemical composition and average coagulation time of the mid-lactation milks were very similar to the corresponding values for the herd bulk milks (Part I, Table 4; Table 1) but the mid-lactation milks had a much greater range of composition and stability. The fifty-five mid-lactation samples were accordingly arranged in groups with increasing coagulation times (10–15, 15–20 min., etc.) and the mean composition of the samples in each of the seven subgroups calculated and compared. None of the group-mean values for the concentration of any constituent exhibited a regular change as the mean coagulation time of the groups increased from 12·0 to 53·8 min. but there were considerable differences in the mean composition of the two extreme groups of milks. The average values for colloidal inorganic calcium, colloidal inorganic phosphorus and soluble inorganic phosphorus were considerably lower in the group of most stable milks than in the least stable group. This suggested that the colloidal inorganic calcium and colloidal inorganic phosphorus content of the caseinate complex might be related to its heat stability. The mean values for these components of the caseinate complex were

therefore calculated (as mg./1 g. casein) for each of the seven subgroups and also the mean values for the other components of the caseinate complex, i.e. magnesium, caseinate calcium, casein phosphorus and citric acid. The variation in the amount of calcium phosphate associated with 1 g. of casein however was not related to the variation in heat stability though the mean value for the most stable subgroup was appreciably lower than that for the least stable subgroup. There was little variation in the mean values for the other components of the caseinate complex.

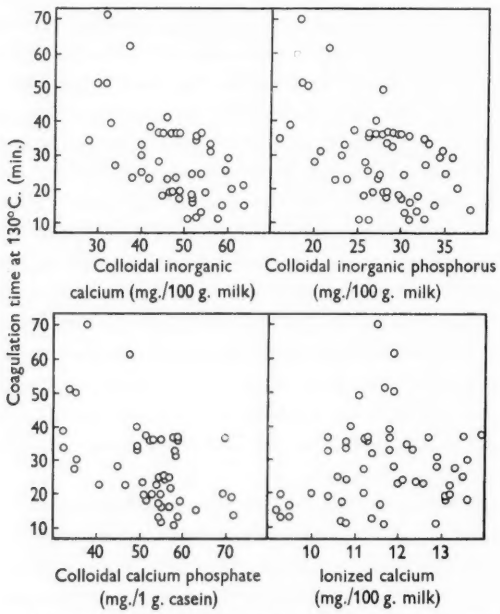


Fig. 3. The relation of concentrations of colloidal inorganic calcium, colloidal inorganic phosphorus, colloidal calcium phosphate and ionized calcium to coagulation time at 130° C. in mid-lactation milks.

The absence of any close relationship between the chemical composition and heat stability of the mid-lactation milks revealed by the preliminary examination of the results was confirmed when the composition and coagulation time of all the individual samples were compared. All that this comparison revealed was that the concentration of colloidal calcium phosphate or of its components, whether expressed per 100 g. of milk or per 1 g. of casein, tended to be lower in the more stable milks and vice versa (Fig. 3). This figure shows also the lack of relation between the concentration of ionized calcium and heat stability. The pH values of the mid-lactation samples were unrelated to their coagulation times.

*Late lactation milk*

The late lactation milks tended to have longer coagulation times than the milks in the other groups (Fig. 1) and abnormalities in chemical composition that might account for this tendency may be seen in Part I, Table 5. The average pH of the late lactation milks was higher than the average for the herd bulk milks, namely, 6·98 as compared with 6·72;

the difference in acidity was also shown by the respective values for titratable acidity. The concentrations of the soluble calcium fractions were low in the late lactation milks. In addition, the late lactation milks contained, on average, less lactose and potassium and more sodium and chloride than the herd bulk milks. There was also a marked increase in the proportion of colloidal calcium, magnesium, citric acid and phosphorus to the soluble fractions of these constituents, compared with the herd bulk milks. However, the increased concentration of casein in the late lactation milks meant that there was only a small increase in the average amount of calcium phosphate associated with 1 g. of casein. The concentrations of the other nitrogenous fractions were also considerably higher in the late lactation milks than in the herd bulk milks but the increased content of lactalbumin plus lactoglobulin, as in the post-colostrum milks, was insufficient to affect adversely the heat stability of the caseinate complex.

Although there was a wide variation in the coagulation times of the late lactation milks, there was no evidence of any relation between chemical composition and heat stability within this group of milks.

*Subclinical mastitis milk*

The milks from cows with subclinical mastitis differed considerably from the herd bulk milks in composition (Part I, Table 6), but they showed no bias towards a lower or higher stability to heat (Fig. 1, Table 1). This implied that the differences in composition between these two types of milk, e.g. in pH and contents of lactose, sodium, chloride, casein, serum proteins and soluble phosphorus, were unimportant in relation to heat stability and that those constituents present in similar concentration, e.g. soluble calcium and soluble magnesium, might be important. Further evidence of the probable unimportance of some of the above constituents was provided by the fact that the subclinical mastitis milks, on average, were less stable to heat than the late lactation milks despite many similarities in composition (Fig. 1; Part I, Tables 5, 6). For example, both were high in pH, sodium, chloride and serum proteins, and both were low in lactose, soluble citric acid and soluble phosphorus. One difference between the subclinical mastitis and late lactation milks was in average casein content, but the lower percentage of casein in the subclinical mastitis milks was associated with a lesser and not with a greater stability to heat such as might be expected.

When the composition and coagulation time of the individual subclinical mastitis milks were compared, little relation was found. Where relationships did exist they were not close and were the opposite of what might be expected. For example, low heat stability tended to be associated with the higher pH values and the lower concentrations of the soluble fractions of calcium and magnesium.

*All milks from individual cows*

Comparison was made also between the composition and coagulation time of all the samples from individual cows so that the whole range of composition and stability was covered simultaneously. As with the group comparisons, only very weak relationships between composition and heat stability were found (Fig. 4). High acidity, as indicated by low pH or high titratable acidity, was usually associated with low heat stability and vice versa. Heat stability tended to decrease as the concentrations of lactose and soluble inorganic phosphorus increased, except with the colostrum samples. There was also some

indication of inverse curvilinear relations between coagulation time and the concentrations of ester phosphorus and ionized calcium. The concentration of none of the other milk constituents nor salt-balance, calculated as described in Part II (p. 263), was related to heat stability.

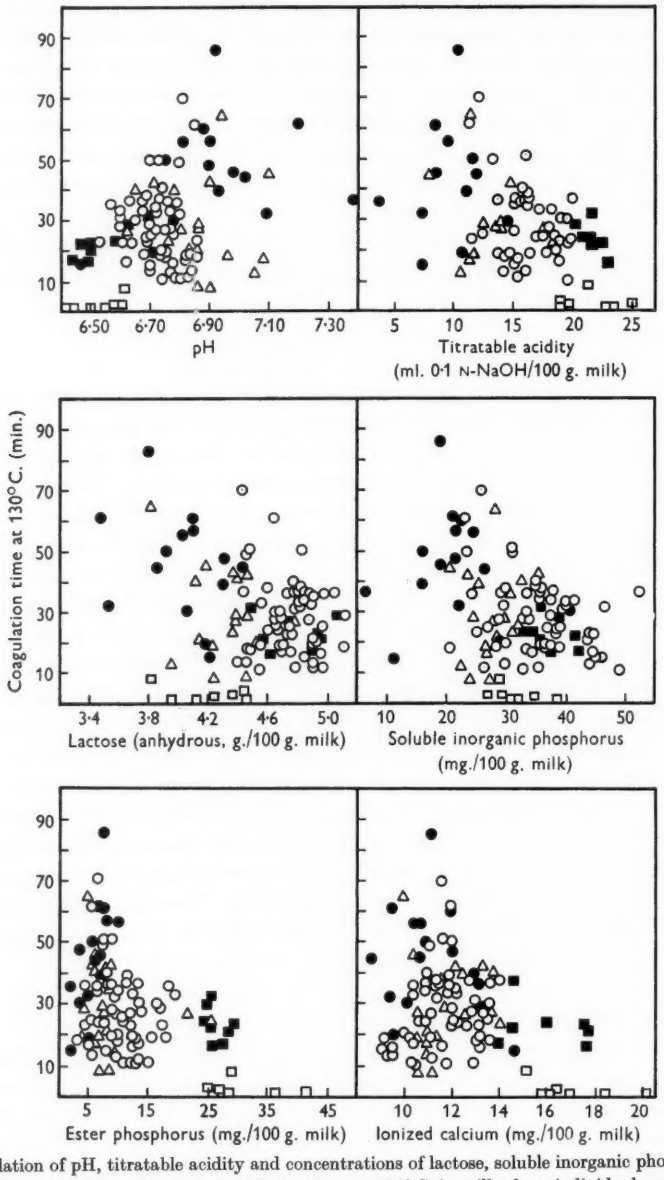


Fig. 4. The relation of pH, titratable acidity and concentrations of lactose, soluble inorganic phosphorus, ester phosphorus and ionized calcium to coagulation time at 130° C. in milks from individual cows in different stages of lactation and from cows with subclinical mastitis. (□, Colostrum; ■, post-colostrum milk; ○, mid-lactation milk; ●, late lactation milk; △, subclinical mastitis milk.)

The variations in the components of the caseinate complex, expressed in mg./1 g. of casein, were also unrelated to the stability of the complex. Since the greatest quantitative variation in the composition of the caseinate complex was in the proportion of calcium phosphate to casein, it was possible that any influence the soluble constituents of milk might have on the stability of the complex might vary with the calcium phosphate content of the complex. To test this hypothesis, the individual cow samples, except colostrum, were first divided into three groups containing milks in which 1 g. of casein was associated respectively with 31·0–49·9, 50·0–59·9 and 60·0–73·5 mg. of calcium phosphate. The milks in the middle group had much the same range of values for the calcium phosphate to casein ratio as the herd bulk milks; those in the first and third groups therefore had ratios respectively lower and higher than average. Within each group, the concentrations of the soluble milk constituents were plotted against the corresponding coagulation times but

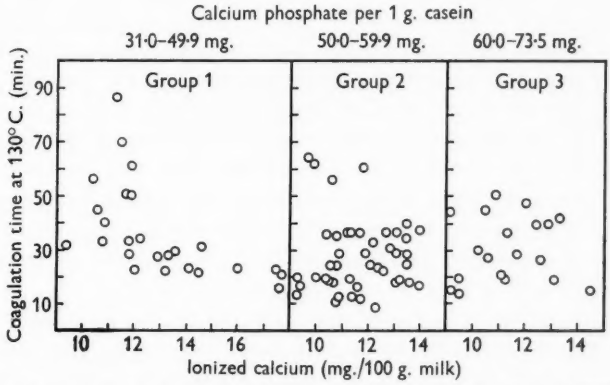


Fig. 5. The relation between concentration of ionized calcium and coagulation time at 130° C. in milks from individual cows grouped according to the amount of calcium phosphate associated with 1 g. of casein.

little relation was found. The results obtained by this procedure for ionized calcium, are shown in Fig. 5. Only with milk in which the caseinate complex was relatively poor in calcium phosphate (group 1) was there any apparent relation between calcium ion concentration and coagulation time. All that this relation indicated was that milks which coagulated in 20–30 min. had calcium ion contents ranging from about 12 to 17·7 mg./100 g. of milk and that more stable milks contained less than 12 mg. of ionized calcium per 100 g., scarcely evidence of a close dependence of coagulation time on the concentration of calcium ions.

In the hope that it might be easier to detect a relation between coagulation time and chemical composition in milks from the same cow, series of milks from each of six cows were examined. But in the six series of samples, the variation in coagulation time could not be related to the variation in chemical composition more closely than in the groups of milks already discussed.

## DISCUSSION

As in previous experiments[1,3,4,5] great variation was found in the stability of milk samples to heat. Coagulation times at 130° C. ranged from 0·6 to 86·2 min., at 140° C. from 0·6 to 35·0 min. and at 150° C. from 0·5 to 7·2 min. With most samples, there was a

linear relation between the logarithm of coagulation time and temperature, a type of relation found also by Webb & Holm (2) and Cole & Tarassuk (1), and by Holm, Deysher & Evans (61) with evaporated milk. However, as noted also by Cole & Tarassuk (1), a significant deviation from a straight line relation was obtained with some samples, usually those whose exact moment of coagulation was difficult to detect. This suggests that attention should be paid to the rate of increase in the particle size of the coagulating caseinate complex, and that a method of assessing heat stability not wholly dependent on visual observation might be preferable. A possible alternative method has been proposed by Whitney, Paulson & Murthey (62), in which coagulation time is obtained by noting when the percentage of filterable nitrogen in heated milk suddenly diminishes.

The slope of the line relating logarithm of coagulation time and temperature was reasonably constant for different milks when their coagulating times were easily determined and indicated that the temperature coefficient ($Q_{10° C.}$) of the coagulation reaction was, on average, 2·7 between 130 and 140° C. and 3·1 between 140 and 150° C. These temperature coefficients are similar to the temperature coefficient ($Q_{10° C.}$) of 3 obtained by Howat & Wright (46) for both the dephosphorylation and coagulation of neutral solutions of calcium caseinate heated at temperatures ranging from 90 to 115° C. for 1–45 hr. Howat & Wright observed that visual coagulation of the caseinate usually occurred when about 45% of the casein phosphorus had been liberated as inorganic phosphate. The variation found in the stability of the caseinate complex in different milks, at a given temperature, indicates either that coagulation is not dependent on a particular degree of dephosphorylation or, less likely, that the rate of dephosphorylation varies from milk to milk.

Except for the colostrum samples, very little relation between heat stability and chemical composition was found despite the wide variation in the coagulation times and chemical composition of the samples. The marked instability to heat of the colostrum samples appeared to be caused, as might be expected, by their very high content of heat sensitive serum proteins. The average concentration of lactalbumin plus lactoglobulin nitrogen was 163·4 mg./100 g., about 2·5 times the average for the herd bulk milks. However, since the post-colostrum and late lactation milks contained, on average, about 35% more lactalbumin plus lactoglobulin than the herd bulk milks and yet were similar or greater in stability than the herd bulk milks, it would appear that there is a critical concentration of serum proteins that has to be exceeded before the caseinate complex becomes abnormally sensitive to heat. The slight overlap in the values for lactalbumin plus lactoglobulin in the individual colostrum and post-colostrum samples made it difficult to decide the critical concentration but it was probably in the region of 100–110 mg./100 g. of milk in terms of nitrogen, or 0·6–0·7% lactalbumin plus lactoglobulin. This estimate agrees reasonably well with the statement of Davies (21) that in the transition from colostrum to normal milk, rapid heat coagulation ceases when the concentration of lactalbumin plus lactoglobulin falls below 0·9%. Below this critical concentration, the amount of serum proteins in the samples was unrelated to coagulation time which is consistent with the observation of Pyne & McHenry (5) that the removal of lactalbumin and lactoglobulin from a mid-lactation milk did not alter its stability to heat.

Several workers have suggested that the stability of the caseinate complex to heat is inversely related to the total calcium content, and especially to the ionized calcium content, of milk. The present investigation did not support these suggestions. There was

a slight indication of an inverse curvilinear relation between calcium ion concentration and coagulation time when all the samples were considered together (Fig. 4) but in the largest single group of milks, those from cows in mid-lactation, no relation was evident (Fig. 3). Also, little relation between calcium ion content and stability was revealed when the variation in the composition of the caseinate complex was minimized by separating the milks into three groups according to the calcium phosphate content of the caseinate complex. Only in the group in which the caseinate complex was relatively poor in calcium phosphate was there an indication of an inverse curvilinear relation between the concentration of calcium ions and coagulation time (Fig. 5). This relation, and the similar one found by Pyne & McHenry (5) with twenty-six mid-lactation milks, would appear to be of little value in predicting the coagulation time of a milk from its calcium ion content. All that either shows is that in a selected population of samples, when the calcium ion concentration exceeds a certain level, about 12 mg./100 g. of milk according to the present results, the milk will be relatively unstable and that below this level, stability will be greater but independent of calcium ion concentration. The fact that the herd bulk milks had a moderate range of values for calcium ion content (10·5–12·8 mg./100 g. milk) and yet had coagulation times ranging from 17·2 to 59·0 min. at 130° C. was further evidence that the concentration of this constituent could not be regarded as a major factor controlling heat stability.

The view of Smeets, Seekles and Boogaerdt (28,29,30,31,32) that milk is unstable to heat when the concentration of calcium ions exceeds about 15 mg./100 ml. of milk ultrafiltrate could not be confirmed. Apart from the colostrum samples, this level was exceeded in only four milks and they were not markedly unstable. The present results did not substantiate the suggestion of Sandelin (25) that the instability of milk, particularly colostrum, is caused by a high concentration of ionized calcium. It is true that the colostrum samples were very unstable to heat and were rich in ionized calcium (15·1–20·1 mg./100 g. milk), but since four of the seven post-colostrum milks had a calcium ion content within this range and yet were relatively stable, it appears that a high calcium ion content is not invariably associated with instability.

There was a slight tendency in the mid-lactation milks for the concentration of colloidal calcium phosphate, and for the calcium phosphate content of the caseinate complex, to be inversely related to coagulation time (Fig. 3). This appeared to support the opinion of Pyne & McHenry (5) that colloidal phosphate is a factor determining the heat stability of milk and suggested that stability might be inversely related to the size of the caseinate micelles; according to Hostettler, Rychener and Künzle (63,64) and Ford, Ramsdell & Landsman (65) the larger caseinate micelles in milk contain more calcium phosphate than the smaller micelles. However, since there was no obvious relation between the composition of the caseinate complex and coagulation time in the other groups of milks, or when the results for all the individual cow samples were examined, it was concluded that the stability of the caseinate complex to heat was not influenced to any significant extent by the calcium phosphate content of the complex or of the milk.

Salt-balance, calculated as described in Part II, p. 263, and believed by Sommer & Hart (3,22,23) to be an important factor in relation to heat stability, was not related to coagulation time in any of the groups of milks or when the results for all samples were collectively examined. This is in agreement with the findings of Holm, Webb & Deysher (4).

In the herd bulk and post-colostrum groups of milks, and when the individual cow samples were considered as a whole (Fig. 4), there was a tendency for pH to be directly related, and titratable acidity to be inversely related to coagulation time. Since these relationships were poor, and the subclinical mastitis group of milks showed the opposite type of relationship between acidity and stability, it was concluded that natural acidity and heat stability were not closely related. This conclusion confirms the earlier reports of Sommer & Hart (3,23) and Holm, Webb & Deysher (4).

It is clear from the preceding discussion that despite the detailed nature of the chemical analysis of the milks in this investigation, no adequate explanation was found for the variation in coagulation times, except with the colostrum samples. The stability to heat of the calcium caseinate-calcium phosphate complex did not appear to be closely dependent on its concentration or on such aspects of its chemical composition as were determined, or on the chemical composition of the aqueous phase in which it was dispersed. The inability to relate closely the chemical composition of the milks to their heat stability suggested either that the analytical methods were insufficiently accurate to detect critical differences in composition or possibly, in view of current work, that the heat stability of the caseinate complex depends partly on some physical or chemical property of the complex not measured in this investigation.

## SUMMARY

1. The variation in the stability of milk protein to heat and the relationship between milk composition and heat stability were examined.

2. The coagulation times of the majority of the milk samples decreased by a factor of about 3 with an increase in temperature of 10° C. over the range 130–150° C. Because of the general proportionality of the coagulation times at 130, 140 and 150° C., the coagulation times at 130° C. only were used as a measure of the stability of the samples to heat.

3. The coagulation times of herd bulk milks ranged from 17·2 to 59·0 min. at 130° C., whereas the range for samples from individual cows was 0·6–86·2 min.

4. Samples of colostrum were very unstable to heat, and milk from cows in late lactation tended to have the longest coagulation times, but otherwise there was little relation between the heat stability of milk and the stage of lactation of the cow.

5. Although colostrum samples were comparatively rich in ionized calcium, their marked instability to heat appeared to be caused solely by their high content of lact-albumin plus lactoglobulin.

6. The stability to heat of the calcium caseinate-calcium phosphate complex in all samples, other than colostrum, could not be closely related either to the concentration and composition of the complex or to the composition and salt-balance of the aqueous phase.

7. When the calcium phosphate content of the caseinate complex was relatively low, the heat stability of the complex tended to be inversely related to the concentration of ionized calcium in the milk, but in general coagulation time was not related to the concentration of ionized calcium.

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# 716. PHYSICAL CHANGES IN MILK CAUSED BY THE ACTION OF RENNET

## I. DESCRIPTION OF APPARATUS FOR MEASURING RIGIDITY MODULI AND INTERNAL VISCOSITIES, TESTS OF RELIABILITY AND SOME OBSERVATIONS ON SYNERESIS

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(With 5 Figures)

### GENERAL INTRODUCTION

Whereas much work has been done on the chemical changes which take place when rennet acts on casein in milk, few quantitative measurements have been made of changes in physical properties.

It is well known that, after adding rennet to milk at a suitable temperature and acidity, no very obvious physical changes take place for some minutes, after which the milk gradually sets into a gel. Later, and especially if the gel is damaged mechanically, whey is expressed and the curd contracts. In making many types of cheese, the curd is cut into very small pieces and, as temperature is raised and acidity increases, so is the whey separated and the curd reduced in moisture content (syneresis).

Many empirical devices have been proposed for following the process of the setting of milk. Among the more quantitative methods may be mentioned the torsion viscometers of King & Melville (1) and, more recently, of Hansson, Sjöström & Samuelsson (2). These latter authors have studied the effects of changes in acidity, fat content, calcium content, etc., and of pasteurization on the rate of setting of milk. They did not attempt, however, to separate the elastic from the viscous deformations, nor immediate elastic reactions from delayed reactions.

The present authors (3) have recently described an apparatus, based on a principle proposed by Saunders & Ward (4), which enables these separate phenomena to be studied independently.

The apparatus is shown in Fig. 1. The details of its working are given in the original paper, but the principle is very simple. Milk, at the required acidity and temperature, with or without added $CaCl_2$, is mixed with the selected amount of rennet and 50 ml. are poured into a U-tube which is immersed in a temperature-controlled bath or thermos flask, shown below the U-tube in the figure. Very small pressures from compressed air can be applied, normally alternately, to the two surfaces of the milk. The milk displaced in the arm not under pressure rises very slightly in the tube and this displacement is measured by the movement of a small 'index' of coloured alcohol in a horizontal capillary tube. When the pressure is released, the milk resumes its level and the index returns to its zero position. The movement of the index magnifies the displacement by about a hundredfold.

As soon as the milk begins to set, much greater pressures are needed to produce any

appreciable displacement of the index. These pressures are increased during the setting process in such a way as to ensure movements of about the same magnitude (0·5–1·0 mm.) in the surface of the milk curd. The recovery of the index now reflects the elasticity of the curd.

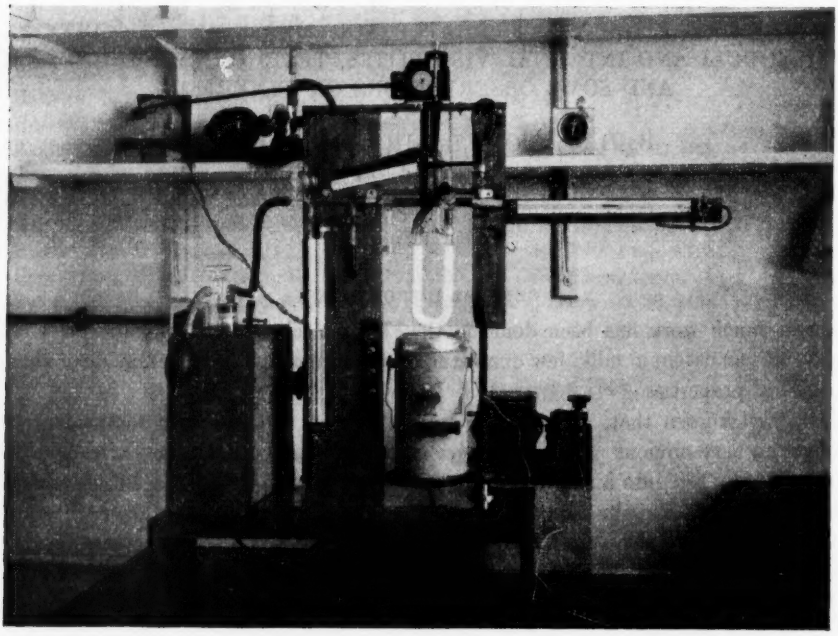

Fig. 1

When the pressure is first applied, the index immediately moves along the capillary. (The capillary must not be too narrow, otherwise the viscosity of the air in it will damp the movement.) After this initial 'jump', the index decelerates so that, by the time the pressure is released after 1 min., it is moving very slowly. On release, there is an immediate elastic recovery, followed by a decelerating movement which is almost (but not quite) complete at the end of a further 2 min. If the recovery is timed, it is found that the logarithm of the proportion of the strain which is unrecovered after $t$ sec. is linear when plotted against $t$, except at the top and bottom of the curve. At the top, the curve must bend steeply upwards to reach $\log 1 = 0$ at zero time and, at the bottom, it turns off asymptotically towards the arbitrarily selected completion time of 120 sec. But the central and linear part of the curve may be extrapolated to distinguish the immediate elastic strain, the lower part giving the retarded elastic strain. A separate rigidity modulus may be calculated for each of these strains, or, as in the present paper, a generalized modulus is obtained from the formula

$$G = PR^4/8La^2h,$$

where $G$ is generalized rigidity modulus, $P$ is applied pressure (dynes/cm.$^2$), $L$ is length of column of curd, $R$ is radius of U-tube, $a$ is radius of capillary tube, $h$ is recovered displacement of index.

The index does not, as a rule, return quite to its initial zero, so we must suppose that there is some very small non-recoverable (plastic) displacement. Some part of this may be due to changes in the shape of the meniscus. There is unlikely to be much slip at the wall, since if this should happen, the curd is shifted bodily in the tube, and the index is liable to rush off the scale.

It will be seen, therefore, that curd, as it forms, behaves as if it consisted of a viscous element (viscosity $\eta_1$), with an immediate elastic element in series (modulus $G_1$); with, again in series, a unit consisting of a viscous element (viscosity $\eta_2$) and an elastic element (modulus $G_2$) slowed down by, and therefore in parallel with it. Such a model is well-known in rheology and is called a 'Burgers Body'. All these constants can be independently determined. Some of their combinations, such as $\eta_2/G_2$, the so-called 'retardation time' ($t_{\text{ret.}}$) and $\eta_1/G_1$, the relaxation time ($t_{\text{rel.}}$) may well prove to be of practical importance. Moreover, the dimensionless ratios, $t_{\text{rel.}}/t_{\text{ret.}}$, $G_1/G_2$ and $\eta_1/\eta_2$ have considerable theoretical interest.

In the present paper, however, we are concerned only with the increase of the generalized modulus $G$ with time of setting. The shape of the $G$-time curve depends on the nature of the milk, its acidity, calcium content, temperature and rennet concentration. In this first paper, experiments are described in which all milks (50 ml.) were acidified by the addition of 0·5 ml. 2% (nominal) lactic acid, 0·5 ml. of 2% standard commercial rennet was added and the setting took place at 32° C. All these conditions will be varied in later experiments.

*Reproducibility of the method*

*Dried milks.* Since samples of liquid milk differ from day to day, a standard, almost fat-free dried milk powder was used to study reproducibility. Since the concentration of free calcium had been diminished in the process of drying, $CaCl_2$ solution was added in

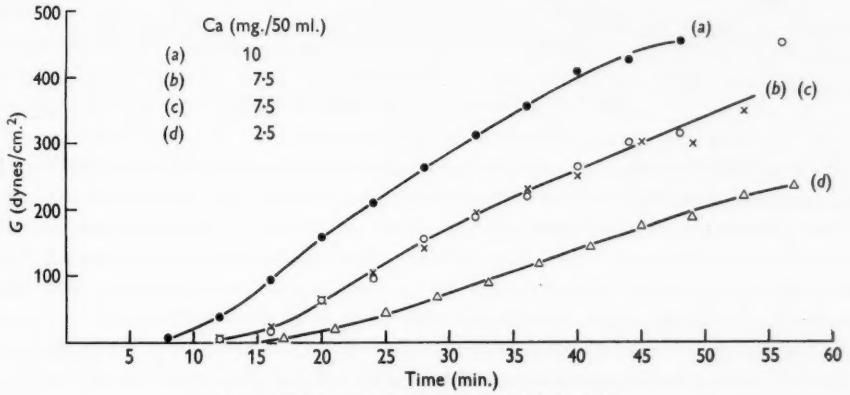


Fig. 2. Fat-free dried milk-effect of added calcium.

various quantities. Fig. 2 shows curves (*b*) and (*c*) for identical composition on different days and curves (*a*), (*b*, *c*) and (*d*) show the effect of varying concentration of $CaCl_2$. It will be seen that, up to a certain time and rigidity, reproducibility is satisfactory in relation to the variations caused by varying the concentration of $CaCl_2$.

For comparison with the degree of reproducibility shown in Fig. 2 (*b* and *c*) curves are shown in Fig. 3 for a number of commercial dried milks from different sources. These are included in order to give some idea of the range of variation for such samples.

For many of these samples, after a certain time the modulus appears to fall and the points soon become quite irregular. Just at this moment, large drops of syneretic liquid can be seen on the walls of the U-tube (see below). Where there is a pool of liquid, the curd is not gripping the wall and there is no wall stress. The wall stress, used in calculating $G$, is given by $PR/2L$. If this figure is used when there is no stress on some parts of the wall, the calculated modulus will be lower than the true modulus. That this is so is easily seen if we imagine the column of curd to be completely detached along half the length of the tube. The stress would then be concentrated along a column only $L/2$ in length and the calculated modulus should therefore be multiplied by 2.

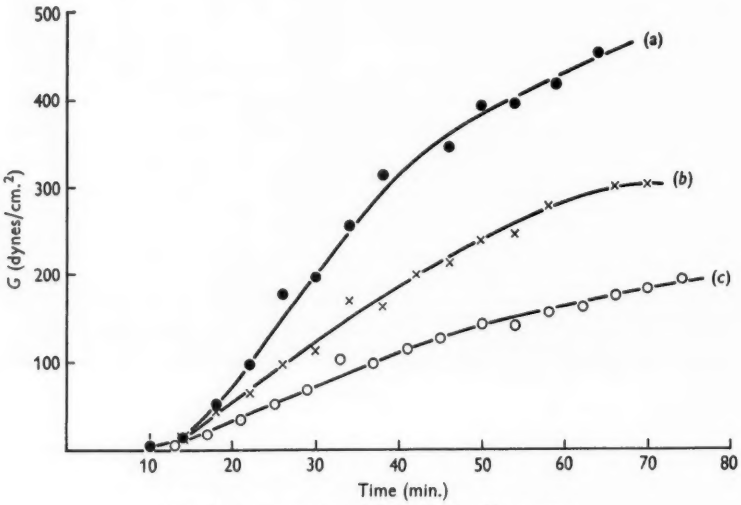


Fig. 3. A comparison of three dried milk samples.

The irregularity of the curve, once syneresis has seriously started, is doubtless due to many causes. Sometimes, points obtained with the pressure from left to right are higher or lower than those obtained with pressure from right to left. This phenomenon must be due to internal stresses in the gel, and it is easily produced by allowing the curd to set with the milk not level in the arms of the U-tube, though it may occur for other reasons, when the curd is level, e.g., when a reading is taken just as the curd starts to set.

*Liquid milks.* Samples of liquid milk cannot be reproduced from day to day and even wider differences are found from month to month. Fig. 4 shows differences in setting-curves for samples taken from cows in the same herd on different dates (*a*, *c*, *d*) and these may be compared with the relatively good agreement shown between one of them (*a*) and the same sample stored in a cold room and re-tested some hours later (*b*). In order systematically to follow progressive changes in milk from a single herd, very carefully controlled bulking procedures and storage conditions are required. It is intended to describe such experiments in a later paper in this series.

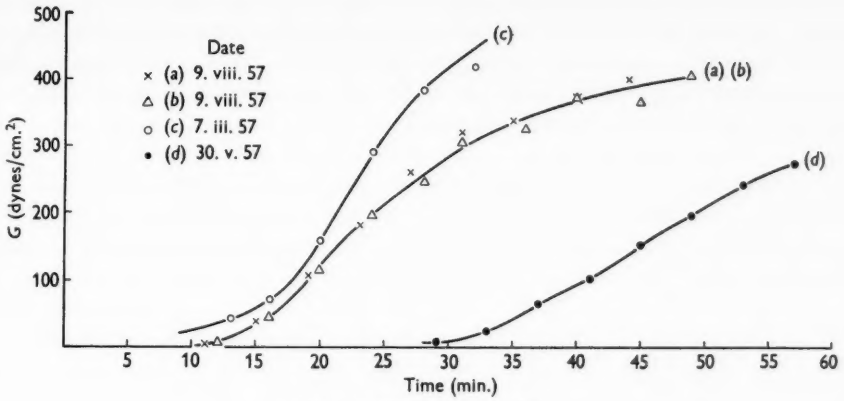


Fig. 4. Samples of liquid milk.

*Interpretations of modulus-time curves*

The curves obtained when $G$ is plotted against time since adding rennet are of a generally sigmoid character, but may be conveniently divided into three parts. For the first few minutes, there is no measurable rigidity; but it must be emphasized that very small rigidities, say $< 10$ dynes/cm.$^2$, cannot be measured in the U-tube apparatus, since liquid milk itself shows a recoil, following very small pressures, due, not to rigidity, but to the levelling of the milk in the two arms of the tube. This effect is much too small to matter once $G$ exceeds about 10 dynes/cm.$^2$. Thus it is not known whether, in the early stages, a very slight rigidity develops or not. Other apparatus must be used to decide this question.

An early writer (Bendixen (5)) has claimed that there is an increase in the viscosity of milk considerably before the 'coagulation time' and that 'it may be possible to anticipate the curd firming by an observation of the viscosity curve during coagulation'. The method of measuring viscosity was not an accurate one, however, and the results are not confirmed by King & Melville's experiments (1).

During the middle part of the setting time, $G$ rises, at first very fast, and later more slowly. Here $G$ is independent of quite large changes in pressure and is unaffected by the frequency of testing. In the final stages, where syneresis has started, $G$ becomes pressure-dependent and, later it is found that frequent testing diminishes $G$, whereas rest periods allow its value to increase (thixotropy).

Attempts were made to fit equations to the sigmoid curves, but the difficulty was always that syneresis started after some time and that the measurements of $G$ then became quite unreliable. Even when a fairly steady value of $G$ seemed to be reached for a time, it was realized that this was probably not a true limiting value.

Fortunately, as will be explained in the next section, methods are gradually being developed which minimize syneresis. Meanwhile, a few dried milks, such as those whose setting curves are shown in Fig. 3*b* and *c*, show little or no syneresis during frequent testing over long periods. It is from the data from such samples that we can get the best idea of what probably happens when curd is allowed to set for a long time without showing visible syneresis.

Relative to the duration of the experiment, the sinusoidal part of the curve is short and not very clearly defined. By far the greater part of the setting process proceeds at a rate which is inversely proportional to the time, in the sense that $G$ plotted against log $t$ gives excellent straight lines except during the first few minutes of setting. One of a number of such curves is shown in Fig. 5.

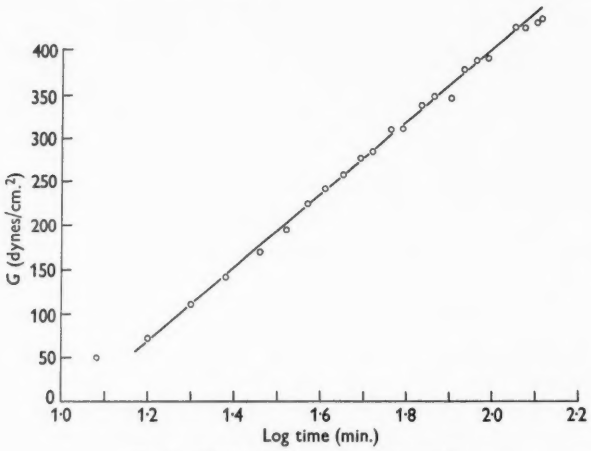


Fig. 5. Dried milk showing no syneresis.

*The phenomena of syneresis*

When milk is set in the U-tube and no moduli are measured, no syneresis is seen for a very long time, except perhaps in the form of very small layers of moisture on the surfaces of the curd. When the curd is subjected to a series of small testing stresses, always in the same direction, syneretic liquid is forced through the curd and appears as a layer of moisture on the surface not under pressure. Very smooth $G$/time curves can be got in this way but are not very meaningful, since the curd is not only becoming firmer but diminishing in moisture content during the experiment.

When the milk is poured down one arm of the U-tube and the curd is tested, in due course, by applying small stresses in alternate directions every 4 min. or so, syneretic liquid collects, after a time, in the arm into which the milk was originally poured. Out of twenty-one carefully watched experiments, this remarkable phenomenon was found to occur 20 times. If the milk is poured simultaneously into both arms of the U-tube, subsequent syneresis appears about equally in both arms: if the milk is injected, by means of a filling tube, into the curved part of the U-tube, syneresis concentrates along the upper part of the curved portion. If the U-tube is subjected to marked vibrations (from the motor-operated cam to be seen at the top of Fig. 1) the syneretic liquid appears to be driven up the arms of the U-tube and tends to collect at the surfaces.

So far, only glass U-tubes have been used; but, if the inside of the tube is etched with hydrofluoric acid, much of the syneresis in the arms of the tube can be avoided. Quite a large pool may sometimes be formed, however, in the bend and this eventually invalidates the calculation of $G$. It was suggested that the glass surface should be saturated with rennet before filling the tube, and that this might prevent asymmetric adsorption when

the milk is poured in, but unfortunately the one-sided syneresis is unaffected by this procedure. A similar experiment pre-rinsing the tube with unrenneted milk was equally ineffective.

Coating the inner walls of the tube with a plastic known to inhibit the syneresis of blood-clots had no apparent effect, nor were matters improved when the plastic was loaded with an inert powder, intended to give a somewhat roughened surface.

It had been suggested that reducing the deformations on the sample might prevent syneresis, since this is almost totally absent when the curd is not strained during setting. However, reducing the displacements to the order of 0·1 mm. had no effect on syneresis and it would be difficult to adapt this type of apparatus to measure strains smaller than this at all accurately.

At the time of writing, the most effective way of preventing syneresis in liquid milk gels would appear to be to roughen the wall of the U-tube mechanically and further experiments on this are in progress.

SUMMARY

A method for measuring the increasing rigidity moduli and internal viscosities of milk during rennet setting is described. Curves are given showing the degree of reproducibility on replicate samples of dried and liquid milks, and of the effects of changes in calcium levels in the case of the former.

A tentative interpretation of the shape of the main part of the setting curve is proposed. The upper part of the curve generally becomes erratic with the onset of syneresis, but some dried milks do not show this behaviour.

Some curious phenomena concerning the production of syneretic liquid (whey) are described and methods for obviating the disturbing effects of syneresis are discussed.

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637.127:536

# 717. PHYSICAL CHANGES IN MILK CAUSED BY THE ACTION OF RENNET

## II. HEATS OF REACTION

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(With 1 Figure)

As a contribution to the present studies (1) of rennet action upon milk, this paper describes a calorimetric investigation of the coagulation and syneresis phenomena.

A recent search of the literature has revealed only one mention of a previous calorimetric examination of this reaction. This was contained in a paper by Coffin & Caule (2) describing the construction and operation of a 40° C. isothermal calorimeter. Their interest in milk, however, was incidental to the main instrumental problems and the measurements were limited and discontinued for a number of reasons (private communication). No heat of coagulation data were given.

### THE CALORIMETRIC METHOD

In the absence of any experimental or calculated heats of reaction, some preliminary experiments with very simple apparatus were carried out which showed that any heat absorbed or evolved during clotting and syneresis must be extremely small and would require highly sensitive apparatus to detect it. The only calorimetric equipment available which seemed at all suitable for this particular reaction has been described by Hirst & Kerridge (3) and was kindly loaned by Dr Hirst for these particular experiments. The calorimeter is capable of measuring quantities of heat of the order of 1–10 cal. Its special feature is that the heat leakage is largely independent of the temperature of the calorimeter and remains constant to within 0·1 cal./hr. for some hours; slow reactions can therefore be followed.

In the present experiments, the calorimeter and its temperature control circuits were as described, except that a potentiometric method of measuring the resistance of the calorimeter thermometer was used in place of the Wheatstone-bridge network. The resistance changes of the 100 Ω platinum resistance thermometer could be measured to a comparable accuracy (0·0001 Ω).

In a typical experiment a sealed, thin glass bulb containing rennet and lactic acid was placed in a retaining cage in the calorimeter vessel and covered with 40 ml. of milk. After the apparatus was assembled and steady conditions reached, the bulb was broken by means of a striker rod, the reactants momentarily stirred and the temperature changes of the calorimeter followed until the rate of change of temperature returned to the initial value. Readings were taken every minute. The system was calibrated by supplying a known amount of heat electrically and the heat of reaction determined graphically by comparison of temperature changes. A typical calibration is shown in Fig. 1 (*b*). Con-

tinuous stirring of the calorimeter contents proved unnecessary and indeed was quite ineffective during rapid clotting. The behaviour of the milk within the sealed calorimeter was judged visually from the simultaneous clotting of a control sample.

### EXPERIMENTS USING COMMERCIAL RENNET

Clotting of fresh, raw, bulked milk (40 ml.) was carried out at 32° C. In the first experiments, following Scott Blair & Burnett(1), 2% lactic acid and 2% rennet were mixed with the milk in the proportion of 1 ml. of each to 98 ml. of milk. Setting to a fairly rigid

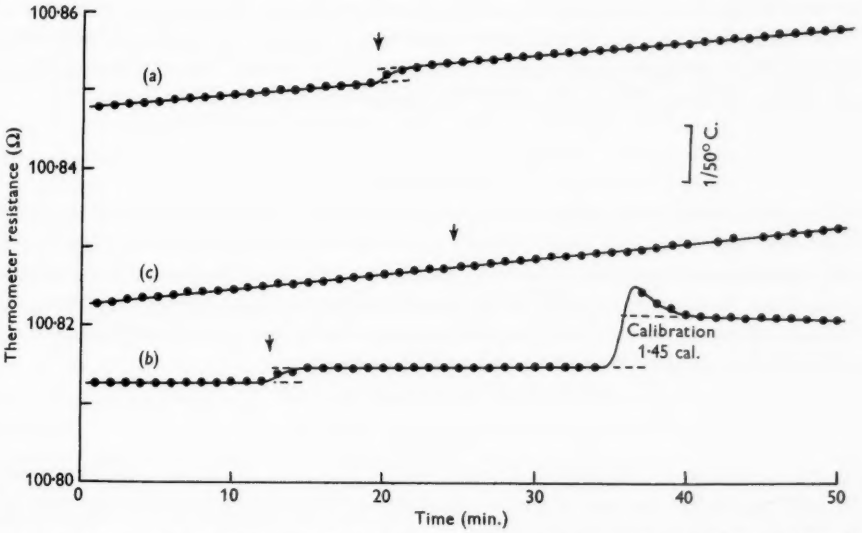


Fig. 1. Heats of reaction of natural, raw milk with lactic acid (2%) and commercial rennet (20%). ↓ Indicates bulb broken. (*a*) Lactic acid and rennet in the bulb. Clotting within 5 min. (*b*) Lactic acid only in the bulb. No clotting. (*c*) Rennet only in the bulb. Lactic acid premixed with milk. Clotting within 5 min.

gel required 20–30 min. No heat of clotting could be detected, however, and the necessity of accelerating the process to improve the chances of detecting any heat change became apparent. This was achieved by increasing the concentration of the rennet to 20%, which led to clotting taking place in 4 or 5 min. The results of typical experiments were as follows, the data around the time of mixing being plotted in Fig. 1. With the lactic acid and rennet initially contained in the bulb, subsequent mixing with the milk resulted almost immediately and before apparent clotting, in an evolution of 0·40 cal. (Fig. 1*a*). Following runs, mixing milk with lactic acid alone (Fig. 1*b*) and acidified milk with rennet alone (Fig. 1*c*) clearly showed that it was a heat of mixing of the lactic acid with milk that was being measured. Results were the same for day to day milk samples. No heat attributable to the clotting or the ensuing syneresis could be detected, although observations were taken for 2 hr. and more after mixing.

The clotting of milk reconstituted from powder gave similar results.

### EXPERIMENTS WITH OTHER ENZYMES

The opportunity was taken of examining the action of trypsin and papain on raw milk.

Trypsin (Armour) was made up with M/50 phosphate buffer, pH 6·8, to a concentration of 80 mg./ml.; 0·5 ml. quantities were used in the experiments. The pH of the milk was adjusted to the optimum of 8·0 with 10N caustic soda. The concentration of papain (B.D.H.) was 40 mg. in 1 ml. of water and was activated with KCN; 1 ml. quantities were used. The milk pH was adjusted to a near optimum of 5·0 with 10N-HCl.

Heat effects upon simple dilution of the enzyme solutions with distilled water were imperceptible. With milk, three runs with trypsin showed exothermic heats of reaction of 0·50, 0·50 and 0·45 cal. which were evolved within 5–8 min. after mixing. Observation of the calorimeter temperature for 2 hr. after mixing showed no detectable heat effect which could be associated with continued digestion of the casein. The results with papain were similar, although the heat evolved was rather lower, about 0·35 cal.

### CONCLUSIONS

The foregoing experiments demonstrate the small heats of action of these enzymes on milk. Whilst the results are useful in setting an upper limit to the heats of reaction, the complicated and obscure kinetics involved prevents any correlation of the data with the enzyme mechanisms. It is apparent too that any future studies would require equipment capable of measurements in terms of milli- or microcalories. Techniques for measurements of this kind on biochemical systems seem well established (4,5).

### SUMMARY

The rennet-catalysed coagulation of milk casein has been examined calorimetrically. The heat of coagulation and syneresis was found to be less than 0·1 cal./40 ml. of milk, the limiting sensitivity of the apparatus.

Heats of reaction approaching 0·5 cal. were observed when the milk was acted upon by the enzymes trypsin and papain. It is doubtful whether, at this stage, the results can be correlated with the several phases of the reaction.

The author is very grateful to Dr W. Hirst of Associated Electrical Industries Ltd., Aldermaston, for the loan of the calorimeter and for his interest in its application. Thanks are also due to Dr G. C. Cheeseman for preparing enzyme solutions and to Dr N. J. Berridge and other members of the Institute staff for useful discussion.

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# 718. RENNETING AND THE CASEINATE PARTICLE: A VISCOMETRIC STUDY*

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## INTRODUCTION

The formation of a gel during the enzymatically induced clotting of milk has been considered generally to involve two separate phases: an enzymatic preliminary phase and a subsequent gelation which is non-enzymatic. Various speculations concerning the chemical and physical processes in each phase have been reviewed by Berridge (1) and Pyne (2). Thus, the first phase has been considered as a simple proteolysis, an alteration of a protective colloid, an alteration of superficial molecules of the casein micelles, and as an enzymatic denaturation. The clotting phase has been regarded as a polyvalent cationic bonding, as thermally-induced denaturation in the presence of calcium ions, and as gelation through crystallite formation.

Viscometric analyses have been used to measure the rate of clotting of milk (3,4,5,6,7) and to gain specific information about the phases of the renneting processes. On the basis of viscometric study of solutions of casein in NaOH, Jirgensons (8) suggested that casein particles decreased in symmetry (i.e. became less spherical) by a type of unfolding during the first phase of renneting. However, Higgins & Hayes (9) found a somewhat decreased intrinsic viscosity in alkaline caseinate solutions following enzymatic action. This result would indicate an increased particle symmetry. Söhngen, Wieringa & Pasveer (10) interpreted a decrease in relative viscosity during renneting as indicative of increasing particle dehydration. According to Hippel & Waugh (11), α-casein exists in a polymerized state at pH values normally occurring in milk and is present as a monomer at pH 12. The present investigation was undertaken in order to relate the viscous changes during renneting with the molecular mechanism of clotting.

## EXPERIMENTAL

Two litres of freshly produced, skimmed raw milk were cooled to 1° C. and brought to a pH of 4·6 by the slow addition of a 1:1 mixture of N/1 hydrochloric and N/1 acetic acids (12). The precipitate was washed four times with similarly cooled distilled water and then three times with absolute ethanol. After a further solution and reprecipitation, the washing was repeated. No drying was permitted, and the isoelectric casein was stored wet in a 1:1 mixture of absolute ethanol and acetone at 1° C. Aliquots of this casein were used to make up the different solutions used in the study.

Casein solutions were prepared as needed by dissolving about 25 ml. of the wet casein in 200 ml. of a cold buffer solution (pH 6·8) of the following composition: 3·56 g. anhydrous $Na_2HPO_4$, 0·91 g. citric acid . 1 $H_2O$, and 5·60 g. KCl, made up to 1 l. with distilled water. The ionic strength is 0·15. Following 5 min. of stirring, the solution was brought to pH 6·8

* This work was done while the author was a Guggenheim Fellow in the Physics Department of the National Institute for Research in Dairying, Shinfield, Reading, England.

with N/1 NaOH and then heated quickly to 80° C. to inactivate native proteases of the milk (13). This process generally removed the alcohol and acetone and brought into solution any undissolved particles. While total solids (and total casein) were being determined, the caseinate solution was stored at 1° C. and then afterwards adjusted to a caseinate concentration of 1% with additional buffer solution.

Two ml. of a 2% rennet solution were added per 100 ml. of caseinate solution. The rennet solutions were made from treated commercial rennet (Chr. Hansen) by diluting 1 ml. of the latter with 49 ml. of the pH 6·8 buffer solution. (Inactive rennet solution was made from the commercial rennet by adjusting the pH to 8–9 with N/1 NaOH for 1 hr. and readjusting it to 5·3 with N/1 HCl. Active rennet solution was given a control treatment with the equivalent final concentration of NaCl.) After 1 hr. of renneting at 25° C. (or 2·5 hr. at 10° C.), during which the relative viscosity of the caseinate solution dropped from 1·42 to 1·38, NaOH solution was added for 1 hr. for rennin inactivation and the pH then readjusted to 6·8 with HCl.

The caseinate solutions were diluted to final concentrations of 0·15, 0·10 and 0·05% with buffer solution or with buffer solution made up with calcium chloride (in a final concentration of 5 mM per litre). These caseinate solutions were then used immediately in the viscosity studies described below.

The method of viscometry followed that of Higgins & Hayes (9). Outflow times were measured in Ostwald-type pipettes of BS design. These were used in water baths at two temperatures: 25·40° ± 0·05° C. and 10°–11° C. (in a refrigerated room). Although the temperature control in the refrigerated room was not precise, temperature recordings were made at each reading to insure equivalence. Moreover, the outflow times of the buffer solution, or the buffer solution with $CaCl_2$, respectively, were obtained with each set of determinations as a measure of solvent viscosity at the respective temperature. The outflow time for water in the pipettes used was about 3 min. at the higher temperature and between 4 and 5 min. at the lower one. The timing clock was graduated in tenths of a second and permitted the estimation of the nearest hundredth. Pycnometer weighings at each temperature gave the respective solution densities. The method of calculation of viscosity increments was that used also by Higgins & Hayes (9) and Hippel & Waugh (11). A partial specific volume of 0·73 was assumed for the alkaline caseinate. (This value was given for different types of casein (14) and falls within the range of values for many types of globular proteins (15).)

## RESULTS

Table 1 presents the reduced viscosities ($\eta_{sp.}/c$) for the three concentrations of caseinate solution under the various experimental conditions. It may be seen that the reduced viscosities are definitely higher at the lower temperature. There is no marked effect of renneting which can be considered as statistically significant. Similarly, the addition of calcium ion apparently has little effect on the reduced viscosity values.

When the values for the reduced viscosities are plotted against concentration, they do not fall upon a straight line. Rather, as Hippel & Waugh (11) have found, the values decrease considerably at the lower concentrations. Hence, it is difficult to make a satisfactory extrapolation of reduced viscosity to the ordinate in order to obtain the intrinsic viscosity. (The sameproblem exists in plotting $ln\ (\eta_{rel.})/c$ against $c$.) The computed axial ratios must necessarily be rough estimates of the statistical mean of the various shapes of the caseinate particles.

Table 1. *Reduced viscosities* ($\eta_{sp.}/c$) *for caseinate solutions of three different concentrations at two different temperatures*

| | 10–11° C. | | | 25·40° C. | | |
|---|---|---|---|---|---|---|
| Sample | 0·15* | 0·10 | 0·05 | 0·15 | 0·10 | 0·05 |
| Untreated casein | 0·26 | 0·24 | 0·15 | 0·22 | 0·17 | 0·10 |
| Renneted casein | 0·26 | 0·25 | 0·15 | 0·23 | 0·18 | 0·08 |
| Untreated casein + Ca | 0·26 | 0·25 | 0·15 | 0·23 | 0·20 | 0·09 |
| Renetted casein + Ca | 0·26 | 0·23 | 0·14 | 0·24 | 0·20 | 0·11 |

* g. casein/decilitre solution.

The intrinsic viscosity of the caseinate particles at 10–11° C. will perhaps lie between the intercepts made by the line of $\eta_{sp.}/c$ 0·15...$\eta_{sp.}/c$ 0·10 and the line of $\eta_{sp.}/c$ 0·10... $\eta_{sp.}/c$ 0·05. These values are, respectively, 0·21 and 0·05 dl./g. According to the method of viscosity increments, with an assumed hydration between 0·3 and 0·4 g. $H_2O$/g. protein, the axial ratios may be derived from Simha's equation (16) or obtained from the tables of Mehl, Oncley & Simha (17). Assuming the particle to have the shape of a prolate ellipsoid, this leads to a length/diameter ratio of 12·2 to 12·8 for the upper limit and 3·8–4·2 for the lower limit. Whereas the lower value is not far removed from that of a sphere, the upper value approaches the lower range of linear macromolecules. As will be seen in the discussion below, the upper range of values is more likely to be correct. For caseinate solutions at 25·40° C., the upper range of the axial ratio is about 9·4–10·3.

## DISCUSSION

As a result of the chemical treatment and the dilution, the caseinate particles studied here are not the casein micelles of milk but more likely much smaller molecules or molecular aggregates of sodium and potassium caseinate (18). Evidence, that casein micelles in native milk are spherical, is provided by electron microscopy (18,19), flow birefringence (20,21), and light scattering (21). The elongated shape of the alkaline caseinates, as proposed from the findings of viscometry (9,11,22,23,24) and indicated by flow birefringence (20,22,25,26), therefore is probably a derivative state. This condition may be due to the method of preparation, which liberates particles of large axial ratio from the native casein micelles or causes an unfolding of previously folded chains.

It is possible that rennet may enzymatically denature the native casein of milk, with a similar production of elongated, unfolded chains, as claimed by Jirgensons (8). However, the decrease in relative viscosity during the initial phase of renneting of milk (3,4,10) does not support such a concept. Decreased relative viscosity or viscosity increment (9) may only be interpreted in terms of decreased axial ratio or decreased particle hydration (17).

In salt solutions, such as N/1 $Na_2SO_4$, sodium caseinate particles have an axial ratio of the order of about 10 (22), similar to that reported here and by others (9,11,23,24). Analysis of the results of Nitschmann & Guggisberg (22) for flow birefringence in the $NaS_2O_4$ solution shows a curve of birefringence against gradient which is concave to the gradient axis. Such a curve may be interpreted to the effect that the caseinate particles behave as rigid rods (27,28). This analysis further supports the concept of an elongated (or unfolded) casein particle. Thus if rennet did act in the first phase by denaturing casein, this effect could not be evident with solutions of sodium caseinate.

Although the present results indicate virtually no change in the viscosity increment as

a result of renneting, those of Higgins & Hayes (9) do show a slight decrease (perhaps due to use of more concentrated rennet solution, higher temperature, and a longer time of reaction). They interpret this change in terms of a decreased axial ratio of the particles. This interpretation is consonant with a presumptive proteolytic action of the enzyme (13,29,30). Some workers have viewed the change as a dehydration effect (cf. (10,31)). However, the mechanism of such an enzymatically induced dehydration has not been explained. Higgins & Hayes (9) believe that their data indicate neither unfolding of polypeptide chains nor any particular degree of proteolytic cleavage.

With respect to the second phase of renneting, this investigation, like that of Christensen (32), shows that added calcium ion causes little change in the viscosity of the caseinate. It seems most reasonable to suggest that since the calcium ion is in adequate supply for clotting, the effect which would be caused by calcium cannot occur here. Two possibilities suggest themselves: that the solutions are too dilute for any particle interaction resembling clotting or that the effect of calcium ion is to promote denaturation, which has already been accomplished by the method of preparation. The viscometric evidence cannot discriminate between these two possibilities.

At the higher temperature, the decrease in viscosity parameters indicates a decrease in hydration of the particles or a change toward a more symmetrical shape. Both phenomena could occur if an aggregation takes place more readily at higher temperatures. Some of the unfolded, straightened, more or less rigid molecules might well associate laterally, in parallel order, their hydrogen bondings being transferred from the less stable molecule-water-molecule association to the more stable one of molecule-molecule. Nitschmann & Guggisberg (26) have also described a decrease in caseinate viscosity due to particle aggregation and a consequent slight increase in symmetry (cf. (33)).

If such a parallel aggregation of linear molecular segments takes place in casein sols, regions of ordered structure ('crystallites') can be formed, connecting regions of less order, as in typical polymeric gels. Berridge (34) believes that denaturation of the casein molecule in renneting would be attended by such a gel network formation at higher temperatures. Hostettler & Imhof (18) have shown a fibrillar organization of casein in the later stages of rennet action. Moreover, the viewpoint of Hippel & Waugh (11) that $\alpha$-casein exists in a polymeric form at pH values below 12 could also fit in with this supposition. Senti, Copley & Nutting (35) have found the typical X-ray pattern of denatured egg white ($X$ $\beta$-keratin) in heat-denatured casein, indicating again molecular aggregation to form crystallites. An X-ray study of rennet-clotted casein should therefore be of some interest in interpreting the mechanism of clotting.

Denaturation of casein in solution at an elevated temperature is a constant difficulty in viscometry. The casein tends to be denatured as an insoluble film which is deposited upon the glass surfaces. Since this removal of casein from solution would be more noticeable with greater dilutions (presumably the molecules tend to accumulate at the surface and hence bring about a proportionately greater drop in concentration with more dilute solutions), it may account in part for the deviation from rectilinearity of the $\eta_{sp.}/c$ against $c$ curves, as was noticed also by Hippel & Waugh (11). It is also possible that at high dilutions there is more particle disaggregation (36). Because of these considerations, it seems valid to use the two higher concentrations here to determine the value of the intrinsic viscosity. The negative slope of these curves, as given by Higgins & Hayes (9) was not found by the writer nor by Hippel & Waugh (11).

SUMMARY

A viscometric study of solutions of alkaline (sodium and potassium) caseinate was made to determine the effects of renneting with and without the addition of calcium ions. There was no real viscometric effect of these treatments at high dilutions. At a higher temperature, the viscosity increment of the solutions decreased. It was suggested that particle aggregation occurs more readily at higher temperatures, leading to a greater particle symmetry as well as causing some dehydration.

The author wishes to thank the members of the staff of the National Institute for Research in Dairying for many helpful suggestions.

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# 719. OBSERVATIONS BY FLUORESCENCE MICROSCOPY OF CASEIN IN MILK, CURD AND CHEESE

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(With 1 Plate)

Although fat globules in milk, which range in diameter from 0·5 to 10 $\mu$, are readily observable under the microscope, a large proportion of casein particles is too small to be seen by any of the methods of optical microscopy. The smallest particles resolvable under the optical microscope are 0·2 $\mu$ with visible light, and 0·1 $\mu$ with ultra-violet (1). An appreciable percentage of casein particles is less than 0·1 $\mu$ in diameter.

For this reason the electron microscope, which can resolve down to 2 m$\mu$, has been used extensively in studies on the size and shape of casein particles (2,3,4,5,6,7). There are, however, disadvantages in electron microscopy: artifacts may arise during fixation, dehydration, shadowing, ultra-sectioning, exposure to high vacuum, or examination under the electron beam; the picture is a static one and the effect of agents producing change in structure, such as coagulation, cannot be seen as a continuous process; in dairy products the fat cannot be seen in relation to the other constituents; polarization effects cannot be studied; and the cost of the equipment is high.

Because optical microscopy has the advantage in these respects, it is, in spite of the fact that it permits observation of only the larger micelles, of considerable value in studies of the size, shape and behaviour of casein particles. It may be used alone or as an adjunct to electron microscopy and such indirect physical methods as flow birefringence. As Cosslett (1) has pointed out, each technique provides evidence of value, giving a partial view of the constitution and structure of the specimen, and the research worker must correlate critically all the clues and deduce a coherent picture.

Earlier microscopic observations of casein used dark-ground illumination (8,9,10,11). The present paper deals with the application of fluorescence microscopy to the study of the appearance of casein in milk, curd and cheese.

## EXPERIMENTAL METHODS

In the Cooke, Troughton & Simms outfit for fluorescence microscopy used in this work, the specimen is illuminated through a deep-blue filter with blue, violet and near ultra-violet light from a mercury vapour lamp. The numerical aperture of the condenser is 1·40 and the iris diaphragm is left fully open. With objectives over $\times 20$ a drop of glycerol or paraffin oil connects condenser and slide. For oil-immersion work a funnel-stop is placed in the objective to reduce the numerical aperture to 1·0 and so diminish glare, and 'Fluoroil' (Edward Gurr, Ltd., London) is used as immersion fluid. A yellow filter is placed in the eyepiece with its thickest plate towards the objective.

Special attention must be paid to cleaning of slides and cover-glasses, since even the slightest contamination may cause a disturbing fluorescence.

Milk is examined after placing a drop on the slide, mixing it with 1 to 3 drops of the fluorochrome solution, applying a cover-glass and leaving for 5–10 min. Curd and cheese are sectioned (10 $\mu$) on a freezing microtome, using 60% gum arabic solution to attach the specimen to the stage. Sections are picked up on the surface of distilled water in a Petri dish. For examination they are transferred to the slide, fluorochromed for 10–15 min. and a cover-glass applied.

## RESULTS

### (*a*) *Milk*

*Fluorochromes for casein in milk.* Among about thirty fluorescent stains tested, the stains specified in Table 1 proved to be suitable for the fluorochroming of casein particles in milk.

The intensity of the uptake of a fluorochrome by casein will depend, among other factors, upon the basic or acidic properties of the side groups and their availability to the molecules of fluorochrome, upon the pH of the medium, etc. The fact that casein contains more acidic groups, which would bind the basic stains, may explain—at least partly—better fluorochroming of casein by basic thioflavine T as compared with related acid stains thioflavine S and primulin.

Table 1. *Fluorochromes for casein in milk*

| Name | Dye group | Basic or acid | Fluorescence colour of casein | |
|---|---|---|---|---|
| Thioflavine T | Thiazole | Basic | Yellowish white | Bright fluorescence |
| Acridine orange | Acridine | Basic | Red orange | |
| Coriphosphine | Acridine | Basic | Red orange | |
| Thioflavine S | Thiazole | Acid | Yellowish white | |
| Primulin | Thiazole | Acid | Yellowish white | Fluorescence less bright |
| Auramine | Phenyl-methane | Basic | Yellowish white | |
| Berberine sulphate | Natural dye | Basic | Yellowish white | |

Mostly thioflavine T or acridine orange were used, in 0·1% solutions (filtered) in Sörensen's phosphate buffer of pH 6·8. Almost similar results were obtained with stains from different sources (G. T. Gurr, Ltd., London, E. Gurr, Ltd., London, Chroma-Gesellschaft, Schmid & Co., Stuttgart-Untertürkheim). When an insight was wanted into the distribution of fat, a 0·1% aqueous solution of phosphine (a variable product; for this work a dark-brown sample made by G. T. Gurr, Ltd., was used) was added in conjunction with acridine orange.

*Fresh milk.* In fluorochromed milk the casein particles appear as minute spherical bodies (perhaps slightly flattened)—with a diameter range from 0·1 to 0·8 $\mu$—in vivid Brownian movement. In fresh milk no aggregates of particles, even small ones, are encountered. With acridine orange the casein particles appear red-orange on a dark background, while the fat globules are transparent green. With phosphine added the fat globules become bright yellowish green (cf. King (12)). View fields under examination quickly develop a greenish glare in the background, the casein particles turn pale-yellow, and the picture loses its initial brightness. Turning to a new view field restores the contrast-rich picture. On adding acridine orange to the milk an excess has to be avoided, otherwise a partial flocculation of casein may take place.

With thioflavine T the casein appears yellowish white, but the fat is not fluorochromed. The background is usually slightly yellowish grey, presumably due to the diffuse

fluorescence of casein particles beyond the limit of visibility. When casein coagulates, by acid or rennet, these tiny particles are entrapped into the clot, and the background becomes black, giving a good contrast to the yellow colour of flocculated casein.

Phase contrast at high magnification also allows the casein particles to be seen, but does not permit the larger casein particles to be distinguished from the smaller fat globules. With thioflavine T only the casein particles are rendered visible.

The casein particles tend in time to stick to any fine scratches on the slide or coverglass, forming linear aggregates of the width of one particle ('bead strings'). Such strings are distinctly nodulated, suggesting an adlineation of spherical particles. In some places the particles may merge together. The casein particles do not attach themselves to the air-milk interface of any air bubbles entrapped in the specimen, as do fat globules whose surface has been hydrophobized in some way. Their surface is thus hydrophilic.

(The data above were presented by the author at a Symposium on Casein, held in the Dairy Research Section, C.S.I.R.O. on 25 and 26 March 1957, shortly before Mohr, Mohr & Drachenfels (13) reported on the fluorochroming of protein (casein) in milk by means of acridine orange.)

The nearly spherical shape of casein particles in the fluorescence microscope (as well as in phase contrast) confirms the picture obtained in the electron microscope by Nitschmann (2), Hostettler & Imhof (3,4), Baud, Morard & Pernoux (5) and others, and eliminates the possibility that it is an artifact. The absence of flow birefringence (Küntzel & Doehner (11), Hostettler, Rychener & Künzle (14), Baud (15)) also supports the concept of the spherical shape of casein particles in milk. As pointed out by Nitschmann (2), this shape is due to surface tension, and not to any special conditions in the mammary gland. A solution of sodium caseinate on addition of calcium chloride contained spherical calcium caseinate particles of a size similar to those in milk.

At medium magnification the fluorochromed casein particles in milk appear dense, with a sharp boundary line around them. However, at high magnification they appear less dense and the transition from the particle to the aqueous medium appears less abrupt. This is in accord with the idea of Küntzel & Doehner (11) that they are made up of loosely aggregated casein molecules (with a hydration degree of about 70%).

The size of casein particles in milk, as determined by the electron microscope, embraces quite a wide range. However, in cow's milk there are always particles below the limit of visibility of the fluorescence microscope (about 0·1 $\mu$). The percentage of particles with a diameter between 0·12 and 0·24 $\mu$ is 37 according to Nitschmann (2), 20 according to Hostettler & Imhof (4) and 10 according to Dyatchenko, Zdanova & Polukarov (7). It has to be taken into consideration that the diameter of hydrated particles, as they occur in milk, is larger by a factor of 1·7 as compared with shrunken particles in an electron microscopic specimen. From data obtained by centrifugal sedimentation, Ford & Ramsdell (16) concluded that in skim-milk about 30% of the total colloidal protein is present as spherical particles 0·17 $\mu$ in diameter, the diameter of the largest particles being 0·35 $\mu$.

*Centrifugal sediment of milk.* There occurs in milk a small proportion of very large casein particles, which can be sedimented by centrifuging at about 900 **g.** for $\frac{1}{2}$ hr. The average diameter of 0·78 $\mu$, as calculated by Hostettler, Rychener & Künzle (14) from the sedimentation velocity, was in good agreement with the value, 0·7–0·8 $\mu$, obtained by direct measurement under the electron microscope by Hostettler & Imhof (4).

Checking these results by fluorochroming the diluted sediment with thioflavine T gave the same average value, 0·8 $\mu$, for the particles, which ranged between 0·5 and 1 $\mu$. These particles were nearly spherical, and in spite of crowding through centrifugation only some few very small aggregates were present. In the supernatant skim-milk the casein particles were appreciably smaller, ranging between 0·1 and 0·5 $\mu$ in diameter.

Separator sludge, fluorochromed with thioflavine T after dilution, also showed a great number of casein particles between 0·5 and 1 $\mu$ in diameter (besides larger greenish yellow bodies, presumably the leucocytes).

*Skim-milk.* In skim-milk from an open separator there may occasionally be seen thin-walled 'bags', presumably the lamellae of collapsed foam (the 'foam membranes' of Hekma and Rahn). The minute fat globules which have escaped the centrifugal force and have been left in skim-milk may be differentiated by fluorochroming with phosphine. They are much less numerous than the casein particles.

*Acid clotting.* On the adding of 0·1 M lactic acid to whole milk, at pH 5·1 only some few small fluffy flocs were visible, whereas at pH 4·8 large dense flocs, consisting of tiny initial corpuscles resulted. The yellow (with thioflavine T) or red-orange (with acridine orange) flocs on an almost black background gave a good contrast. These data agree with the electron microscopic observations of Hostettler & Imhof (4) that at pH 5·0 almost no coagulation takes place, whereas at pH 4·8 large flocs are formed.

According to Wiegner (9) the larger casein particles act as nuclei for coagulation, the smaller particles clinging to larger ones. Hence, the small particles invisible in the fluorescence microscope are entrapped into the clot, and the diffuse greyish background, as obtained with thioflavine T, turns almost black. Examination in the fluorescence microscope allows the coagulation phenomena in milk and milk products (sterilized milk, frozen milk, condensed milk, frozen condensed milk, etc.) caused by heating, freezing, homogenizing, bacterial action, etc. to be followed from the very first stages. It is more sensitive than qualitative tests involving macroscopically perceivable flocculation (boiling test, alcohol test, alizarol test, Storrs test, etc.).

*Rennet clotting.* Renneting was carried out in a test tube; small samples were fluorochromed on the slide and gently pressed flat with a cover glass. About 15 min. after adding the rennet no flocculation was visible with the naked eye. Under the fluorescence microscope, however, numerous small flocs were present. The flocs exhibited a very fine granular structure, suggesting an adjoining but no coalescence of the original particles. Whereas the single particles still left behind were in vivid Brownian movement, the particles in the flocs were stationary. About 30 min. after adding the rennet, when flocs began to appear on the walls of the test tube, under the fluorescence microscope very numerous flocs of different sizes were present. In the larger flocs fat globules were entrapped. The flocs tended to take a stretched shape, forming a distinct network pattern. The picture was very similar to that obtained by Imhof & Hostettler in the electron microscope (Figs. 2 and 3 in (17)). Although the basic material of the network was finely granulated, some very thin fibres were present, particularly in places where the flocs had been stretched by pressure on the cover-glass. In the later stages of clotting very large lumps of finely granulated curd with cavities containing serum and fat globules were present. The coagulated casein did not seem to stick to the fat globules, which accords with the fact that care must be exercised in the cutting of rennet clot and during the initial agitation in the vat, in order to avoid loss of fat into the whey. On the other hand, the

fat globules tended to coalesce with each other. Generally, no single particles or flocs of casein occurred in the cavities. However, small flocs did occur in the serum around the lumps, apparently being torn off from the coagulated mass. When the handling of curd in cheese vat during cutting and agitation is not careful enough, such detached casein flocs ('Käsestaub') may cause loss of casein into whey.

The electron microscopic observations on renneting are in general agreement with the above. According to Baud, Morard & Pernoux (18) the adlined casein particles formed nodulated fibrils by means of thin fibrous annexes. Subsequently the fibrils adjoined into strings, which finally formed a three-dimensional network. The formation of network was also reported by Hostettler, Stein & Imhof (19), Imhof & Hostettler (17), and Peters, Dietrich & Hansen (20).

### (*b*) *Curd*

The freezing-microtome sections of curd (10 $\mu$ thick) were in most cases fluorochromed with thioflavine T or with a mixture (2:1) of acridine orange and phosphine. With thioflavine T the casein appeared yellow or greenish yellow on a black background, while the fat did not display any fluorescence. With thioflavine S and primulin the casein fluoresced greenish yellow and the fat slightly transparent green on an almost black background. With acridine orange and coriphosphine the casein was red-orange and the fat transparent green on dark background (curd fluorochromed with acridine orange on prolonged illumination did not lose its bright colour, as was the case with milk). The fat fluoresced with phosphine yellowish green.

The curd grains from Cheddar cheesemaking, about 45 min. after cutting, were surrounded by a distinct surface film ('skin'), which contained only few fat globules. The casein in the grains showed granular structure; however, overspanning large cavities there occurred some few thin nodulated fibres.

Just before the removal of whey, the casein still appeared in the granular state, whereas the fat globules were slightly coalesced. In cheddared and milled curd there was pronounced formation of thin and long fibres. The fibrous pattern was best perceivable with thioflavine T, whereas the mixture of acridine orange and phosphine was of advantage in showing the distribution of fat.

Cheddared curd from the cheddaring machine of Czulak & Hammond (21) behaved differently in sections cut parallel to the direction of macroscopically perceivable fibre and transversally to this direction (provided a pronounced formation of the so-called 'chicken-breast texture' took place). In the longitudinal sections numerous fine, long threads (micro-fibres), 0·1–0·5 $\mu$ thick, 10–50 $\mu$ long, were present forming a network with wide meshes, more or less stretched in the direction of the macro-fibre. The picture resembled, to a certain degree, an electron micrograph of coagulated blood fibrine by Wolpers & Ruska (22). Occasionally there occurred networks made up of coarse fibres, which in turn consisted of entwined thin fibres (Pl. 1, Fig. 1). In the transverse sections the micro-fibrous structure was not so conspicuous and the basic material seemed to be predominantly in a granular state (Pl. 1, Fig. 2).

When the formation of macro-fibrous texture on machine-cheddaring was less pronounced, the micro-fibres were still very thin, but shorter, and the meshes of the network were smaller. With poor development of macro-fibrous texture only a few micro-fibres were present, the casein being in granular form. Hence, the fibrous texture of curd

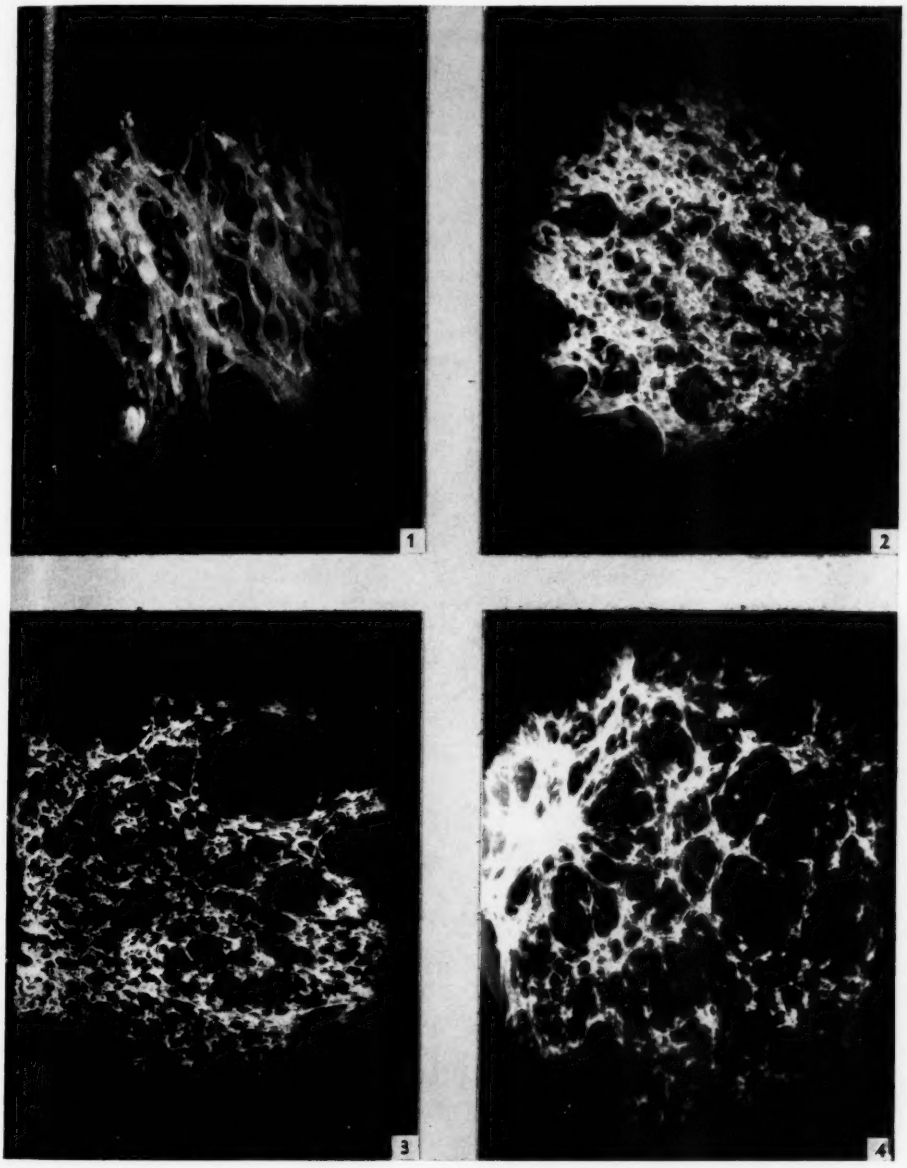


Fig. 1. Stretched network of coarse casein fibres in curd cheddared in the machine of Czulak & Hammond. Section along the direction of macro-fibres. Fluorochroming with acridine orange: casein red orange, fat green. Magnification about ×300.

Fig. 2. Predominantly granular structure of casein in machine-cheddared curd. Section across the direction of macro-fibres. Fluorochroming with acridine orange. Magnification about ×300.

Fig. 3. Transition of granular form of casein into fibrous form in spread-out section of curd. Fluorochroming with thioflavine T: casein greenish yellow, fat non-fluorescent. Magnification about ×300.

Fig. 4. Pronounced fibrous network of casein in spread-out section of curd. Fluorochroming with thioflavine T. Magnification about ×350.

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attained on a properly carried out cheddaring extends from the macroscopical dimensions down to the limit of visibility in the fluorescence microscope. The question how the molecules of different modifications of casein are arranged into the micro-fibres, or whether there exist smaller subunits than micro-fibres, remains for further study.

The casein, either in granular or in fibrous form, did not show any tendency to adhere to the fat, or *vice versa*.

The sections of curd on the surface of distilled water (in the Petri dish) on standing slowly spread out. On holding the sections for about 5 days in the refrigerator the increase in area was four- to tenfold. The phenomenon occurred with sections up to 10 $\mu$ thick, but not with sections 20 $\mu$ thick. The boundaries of curd grains, easily perceivable in fresh sections under a magnifier, disappear on spreading-out of the sections. Sections across the direction of macro-fibres in cheddared curd, or sections from a curd without fibrous texture (cut at random) expanded both in their width and length (with time the corners becoming rounded). However, sections cut along the direction of macro-fibres in a cheddared curd with very pronounced macro-fibrous texture, expanded in their width, and only very slightly in their length.

Under the fluorescence microscope, in spread-out sections of curd with well developed fibrous texture, the length of the micro-fibres has increased appreciably, from 10–50 to 100–150 $\mu$, the size of the meshes increasing accordingly, whereas the width of fibres remained unchanged (0·1–0·5 $\mu$). The fibres were of uniform width over their entire length and without nodulation. At times it could be observed that bundles of threads were drawn out from the granular material in the 'knots' of meshes, and split into very fine fibres. Thus, the transition of casein from granular (globular) state into fibrous can be followed directly under the microscope (Pl. 1, Fig. 3). A practical measure of this property of casein, important in Cheddar cheese making (also in making Caccio-cavallo cheese), is the length of threads in the hot iron test. A preliminary microscopic examination of these threads disclosed them as bundles of very thin fibres.

Even in sections of cheddared curd with poorly developed fibrous texture and with the casein overwhelmingly in the granular state on spreading-out on water, a network of thin, long fibres appeared with some granular not-disentangled material still left behind in the 'knots' of meshes (Pl. 1, Fig. 4).

The fibrous network in a specimen of a spread-out section in about half an hour begins to collapse, the torn-off fibres curling up and becoming shorter and thicker.

Sections about 20 $\mu$ thick originating from curd displaying no macro-fibres, after being held for 5 days in the refrigerator, did not spread out and retained their granular structure (with a slight formation of short fibres in some few places).

Electron microscopic studies on changes in the structure of rennet curd during acid development have been made by Imhof & Hostettler[17,23]. To this end, the fixed and dehydrated specimens were mounted in a polymer of methacrylic acid esters and cut by means of the ultra-microtome into sections 0·1–0·2 $\mu$ thick. However, dehydration with alcohol removes most of the fat, and no information regarding the distribution of fat in the curd may be obtained. Upon acid development in curd (the pH dropping from 6 to 5) the casein particles coalesced to compact threads. However, these threads under the electron microscope were appreciably thicker and shorter than the micro-fibres described above, and their width was not uniform.

### (*c*) *Cheese*

The freezing-microtome sections of cheese (10 $\mu$ thick) were fluorochromed with one of the following solutions: (1) a mixture (1:1) of basic fuchsin and phosphine, both 0·1% solutions in 75% glycerol (cf. King(12)); (2) a mixture (2:1) of acridine orange and phosphine; and (3) thioflavine T. With the first the proteinaceous material fluoresces deep-red, with the second red orange, and with the third yellow or greenish yellow. With thioflavine T the cavities filled with fat and/or free water appear as black voids in the sponge-like pattern of proteinaceous matter.

In the present study only Cheddar, Emmental, Edam and processed Cheddar cheese were examined. The grain boundaries (well visible also under a magnifier) were present in the first three cheese types, but were absent in the last one. The boundary layers generally contained only few fat globules, and exhibited the intense fluorescence colour of protein, while there were abundant fat globules, slightly coalesced, inside the grains. The protein in the boundary layer as well as inside the grain was in the granular state. The layer was about 10–20 $\mu$ thick, and sharply differentiated against the interior of the grain (particularly in Emmental cheese). After some standing of specimens, cracks tended to develop along the layers (the layer being split into two halves), and sometimes free fat filled the crevices.

On standing, the micro-sections (10 $\mu$ thick) of Cheddar, Emmental and Edam cheese on the surface of distilled water slowly spread out, whereas the sections of process cheese in a short time disintegrated into small particles, which sank to the bottom of the Petri dish. The spread-out sections under the fluorescence microscope displayed the conversion of granular pattern into a fibrous network (best depicted by using thioflavine T). However, most of the fibres appeared plump as compared with the slender fibres in curd.


## SUMMARY

Fluorescence microscopy was applied to the examination of casein in milk, curd and cheese, to check or support the picture obtained by electron microscopy. The best fluorochroming effect was shown by thioflavine T, acridine orange, coriphosphine and basic fuchsin.

In milk, larger casein particles (0·1–0·8 $\mu$ in diameter) were rendered visible as minute nearly spherical corpuscles in lively Brownian movement. The sediment obtained on centrifuging the whole milk at about 900 g contained predominantly large particles (average diameter 0·8 $\mu$). On acid or rennet coagulation the casein particles aggregated to flocs, which finally formed a three-dimensional framework.

From curd or cheese freezing-microtome sections (10 $\mu$ thick) were examined. The curds which did not possess the fibrous ('chicken-breast') texture, characteristic for well-cheddared curd, under the fluorescence microscope displayed a granular structure, whereas curds with a pronounced fibrous texture showed a network of thin micro-fibres, particularly in sections cut along the direction of macro-fibres.

The sections (10 $\mu$ or less thick) of curd and cheese on the surface of water slowly spread out. The spreading was accompanied either by conversion of the form of casein from granular into fibrous, or by stretching of the micro-fibres in the already existing network.

The grain boundaries in curd and cheese (Cheddar, Emmental, Edam) were almost free of fat. No grain boundaries existed in process cheese.

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# 720. A RAPID OPTICAL TEST FOR HOMOGENIZER EFFICIENCY

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(With 2 Figures)

## INTRODUCTION

A number of optical methods have been proposed recently for testing the efficiency of homogenizers (1,2,3), and in view of the current interest in the homogenization of milk (4), a further spectroturbimetric test has been devised. The method proposed here is an improvement on those already published, since it enables milk samples to be examined without dilution or addition of reagents. This feature should enable the method to be adapted to continuous recording.

The transparency of milk depends upon the radiation scattered by both the protein and the fat components. In more detailed studies of the light transmission of milk (5,6), it has been shown that the turbidity at long wavelengths is almost completely due to the fat globules. Since the turbidity is a function of both the fat globule size and concentration, it can be seen that the transparency of a milk sample will depend upon both the degree of homogenization and the fat content.

In previous optical methods, the milk samples were diluted with large volumes of water and were examined in cells with path-lengths of the order of 10 mm. The use of very thin absorption cells (path length *ca.* 0·1 mm.) enables undiluted milk samples to be examined in the near infra-red region and, at the same time, avoids the large energy loss due to strong water absorption (7).

## DISCUSSION OF PREVIOUS OPTICAL METHODS

Deackoff & Rees (3) have used a method developed from the original Ashworth method (1), in which the turbidity due to the protein was eliminated by allowing the diluted milk sample to stand for 30 min. in the presence of alkali. A standard commercial spectrophotometer was then used to measure the transmission at 1·02 $\mu$; the transmission values being dependent upon the fat content of the milk. The method of Lagoni & Merten (2) provides an index '$x$-value' which is independent of the fat content, but requires measurements to be made at a number of different wavelengths on diluted milk samples.

The method outlined below is dependent upon the fat content to about the same extent as the Deackoff & Rees method and should prove of particular value in the examination of bulk milk samples where large changes in fat content are not expected.

## EXPERIMENTAL

Milk samples used for testing this method were pre-heated to 70° C. (160° F.) and were homogenized in a Rannie Homogenizer (22 gal./hr. capacity) at 210, 140 and 70 kg./cm.$^2$ (3000, 2000 and 1000 lb./sq.in.).

Transmission measurements were carried out using a Unicam SP 500 Spectrophotometer in which the wavelength range had been extended to 2·6 $\mu$ (8). The milk samples were transferred directly into glass absorption cells of the type normally used for infrared spectrophotometry, their path-lengths being measured by interferometric methods. For the preliminary experiments, the detector unit was placed about 25 cm. away from the absorption cells, to ensure that only a small angle was subtended by the detector at the sample. This precaution was not found to be necessary when examining homogenized milk samples only, since the smaller fat globule size permits the use of a larger collecting angle for the detector. In the normal cell position the distance between the cell and detector was about 6 cm.

Measurements were made on the same milk samples using two other spectroturbimetric methods. The Deackoff & Rees method was carried out as described (3), the diluted milk samples being examined at 1·02 $\mu$. In the case of the Lagoni & Merten method, the '$x$-value' was obtained from the slope of the log (optical density)/log (wavelength) relationship over the wavelength range 0·35–1 $\mu$.

## RESULTS AND DISCUSSION

Fig. 1 shows the transmission of a milk sample (3·80% fat, 8·89% S.N.F.) before and after treatment at various homogenization pressures.

Fig. 2 shows the effects of homogenization, as measured by the three different methods on the same milk samples. In each case, the points at the left of each curve represent the values for unhomogenized milk. The transmissions at wavelengths of 2·2$\mu$, 1·7$\mu$ and 1·02$\mu$ are shown for the undiluted samples.

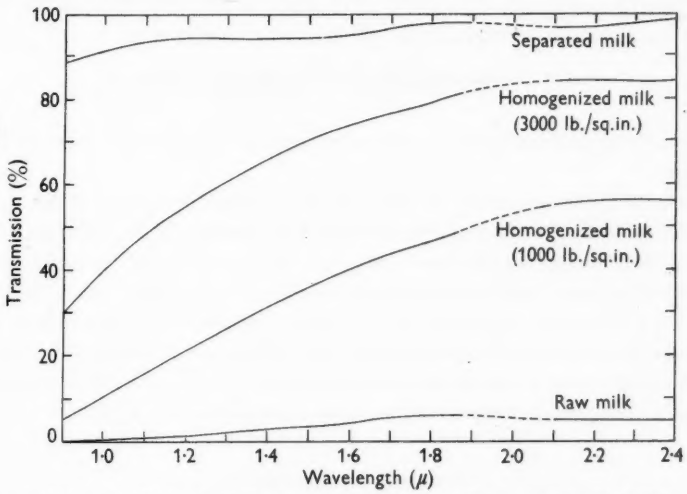


Fig. 1. Transmission of radiation by milk samples in 0·135 mm. cells, compared with 0·135 mm. water. – – –, Indicates regions of strong water absorption.

From these curves, it can be seen that the Deackoff & Rees method is the most sensitive to homogenization conditions in the region of higher pressures, i.e. those used in commercial practice. The results for the undiluted milk samples do not appear to be quite as

sensitive to changes in homogenization conditions, but are considerably more sensitive than those of the Lagoni & Merten method. Multiple scattering effects are probably responsible for the lowered sensitivity of the method using undiluted milk samples.

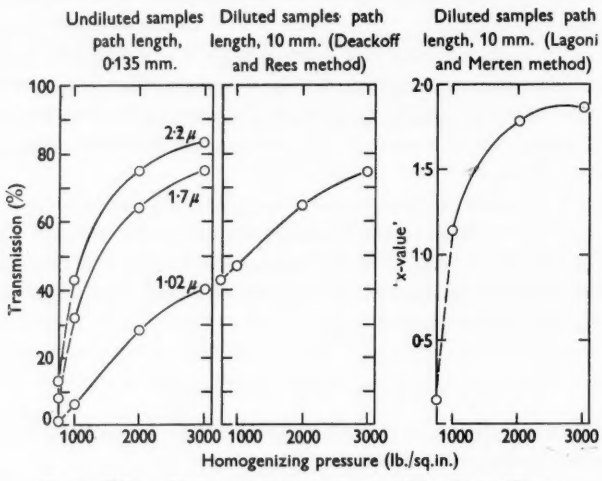
Undiluted samples path length, 0·135 mm.
Diluted samples path length, 10 mm. (Deackoff and Rees method)
Diluted samples path length, 10 mm. (Lagoni and Merten method)
Transmission (%)
'x-value'
2·2 μ
1·7 μ
1·02 μ
Homogenizing pressure (lb./sq.in.)

Fig. 2. Effect of homogenization, as measured by three different spectroturbimetric methods.

## EFFECTS OF CHANGES IN FAT CONTENT

In the Ashworth method (1), the effects of changes in fat content were allowed for by expressing the results as a constant ($K$) given by the relationship:

$$K = \frac{\text{optical density}}{\text{fat \%}} = \frac{\log 1/T}{\text{fat \%}},$$

where $T$ is the percent transmission. A similar method could be used with the Deackoff & Rees method.

In order to test the sensitivity of the present method to changes in fat content, the transmission of a sample of homogenized milk was measured after dilution with known small volumes of water. An effective change in fat content from 3% to 4% gave rise to a change in reading at 1·7 and 2·2 $\mu$ of less than 3%; a similar change using the Deackoff & Rees method gave rise to a change of 5% in the transmission value. These values are slightly dependent upon the path length of the cell and the wavelength used, but it is considered unlikely that a change in fat content from 4 to 2% will affect the transmission as much as a change in homogenizing pressure from 210 to 140 kg./cm.$^2$.

## FURTHER DEVELOPMENTS

In view of the close similarity of the curves for 1·7 and 2·2 $\mu$, it can be seen that the choice of wavelength used for this method is not critical and it should be possible to construct a simple infra-red colorimeter for routine control purposes. A combination of a tungsten lamp source with a photoconductive cell detector should prove adequate when employed with a silicon filter to remove wavelengths less than about 1·1 $\mu$. The longer

wavelengths would be removed by water absorption in the samples. Flow-through type glass absorption cells would enable the instrument to be used for continuous monitoring of homogenized milk samples.

SUMMARY

It has been shown that the degree of homogenization can be tested directly by measuring the transmission of milk samples of thickness *ca.* 0·1 mm., using wavelengths in the near infra-red region.

Thanks are due to Miss M. A. Edwards for preparing the homogenized milk samples and to Miss J. Kingston for assistance in carrying out the transmission measurements.

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# 721. AN ANALYSIS OF THE PERFORMANCE OF AN ULTRA-HIGH-TEMPERATURE MILK STERILIZING PLANT

## II. CALCULATION OF THE BACTERICIDAL EFFECTIVENESS

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(With 9 Figures)

The purpose of this paper is to show how the performance of a continuous heat-treatment plant may be predicted from the temperature distribution and the flow time distributions obtained as described in the first paper of this series (1), using thermal death-time characteristics appropriate to the micro-organisms of greatest importance. The calculations are carried out using the data obtained for the plant described in the previous paper, and they provide an estimate of the bactericidal effectiveness to be expected from that plant.

The problem considered here is not unlike that in the canning industry in determining the integrated lethal effect of the heat treatment of a canned product. The literature on this subject extends over more than thirty years (e.g. (2,3,4)). However, it appears to the writer that the characteristics used in the theory as developed in the canning industry, for example those denoted by the symbols $F$, $z$ and $Z$, are highly arbitrary. Their use does not lead to the easiest way of considering the problem. The characteristics having been defined once when the basis of the theory was laid down, subsequent workers have, with a laudable and unusual desire for standardization, retained the original conceptions and added others as the need arose. The result is a complex theory which is difficult to follow by anyone not well versed in the basic ideas.

There has been little application of this theory to milk processing other than a pioneer paper by Ball (5). There seems, therefore, nothing to be gained by attempting to follow the principles developed in the canning industry. A different approach will be used which seems to be in some ways a simplification, and which is more convenient for the particular problem under consideration.

### LIST OF SYMBOLS

$L$ = logarithm to the base 10 of the proportion of surviving organisms in a population.

$t$ = time (general symbol).

$f(t)$ = general symbol for a function of time $t$.

$T$ = total time of flow through heating section.

$K_\theta$ = slope of a thermal death line, linear on logarithmic co-ordinates, at a temperature $\theta$.

$\theta$ = temperature in degrees Celsius.

$N$ = number of organisms.

$R$ = proportion of surviving organisms in a population, for the holding section of sterilizing plant.

$S$ = proportion of surviving organisms in a population, for the heating section of sterilizing plant.

$H(t)$ = holding time distribution function, obtained experimentally.

$J(T)$ = heating time distribution function, obtained experimentally.

### THERMAL DEATH CHARACTERISTICS OF ORGANISMS

It has been widely assumed that the order of thermal death of micro-organisms is logarithmic and this assumption is implicit in the theory used in the canning industry. Since it simplifies the mathematical analysis it will also be used here. There is, however, reason to suppose that a logarithmic order of death is not general, so that some more complex relation is necessary. It is still possible to deal with some non-logarithmic orders of death by an application of the logarithmic theory, and this will be considered in the Appendix.

If the thermal death of a bacterial population is expressed in the form of a graph of (logarithm of the fraction of surviving organisms) against (time of heating) for a constant treatment temperature, then on the assumption of a logarithmic order of death a straight line will be obtained (Fig. 1). This straight line will pass through the origin, and will be described by an equation of the form

$$L = -K_\theta t. \tag{1}$$

$K_\theta$ is a constant which depends on the treatment temperature. It is the change in $L$ for a unit change in time. The minus sign is included in the equation so that $K_\theta$ is always positive, in spite of the fact that $L$ is always negative. It follows that $K_\theta$ is the inverse of the decimal reduction time at the temperature, $\theta$, since the decimal reduction time is the time for a unit change in $L$, or for a change of a factor of 10 in the fraction of survivors.

As the temperature increases, so does $K_\theta$. This is shown in Fig. 1, for $\theta_3 > \theta_2 > \theta_1$. The relation between $K_\theta$ and $\theta$ is given most generally by Arrhenius's equation, which can be expressed as

$$\log_{10}(K_\theta) = \frac{A}{\theta + 273} + B, \tag{2}$$

where $(\theta + 273)$ is the absolute temperature corresponding to $\theta°$ C., and $A$ and $B$ are constants. If $\log_{10}(K_\theta)$ and $1/(\theta+273)$ are plotted for several corresponding values of $\theta$ and $K_\theta$ obtained by experiment, a linear relation is obtained. From this relation, values of $K_\theta$ for other values of $\theta$ can be determined. Generally, the other values of $\theta$ will be outside the range of known values, so that extrapolation is necessary. It is probable that a reasonable degree of extrapolation is justifiable. For example, if values of $K_\theta$ are obtained by experiment for values of $\theta$ between 110° and 120° C., extrapolation to values of $\theta$ of 135° C. is probably justifiable. Some very marked change in the kinetics of the thermal death process would be necessary to introduce serious errors over this range.

Another method of expressing the variation of $K$ with temperature is by means of the ratio of the values of $K$ at different temperatures. It can be shown that the ratio of the value of $K$ at any temperature to its value at a temperature 10° C. higher is constant,

over a restricted range of temperature. For convenience, this ratio is often called the $Q_{10}$ of the thermal death process. Therefore

$$Q_{10}=\frac{K_{\theta+10}}{K_\theta}. \tag{3}$$

A 10° C. interval for the ratio of the thermal death-time characteristics is inconveniently high in the present work. An alternative ratio

$$Q_1=\frac{K_{\theta+1}}{K_\theta} \tag{4}$$

is therefore used to define the increase in the slope of the thermal death line for a 1° C. rise in temperature. It follows that $Q_1$ and $Q_{10}$ are related by

$$Q_{10}=Q_1^{10}. \tag{5}$$

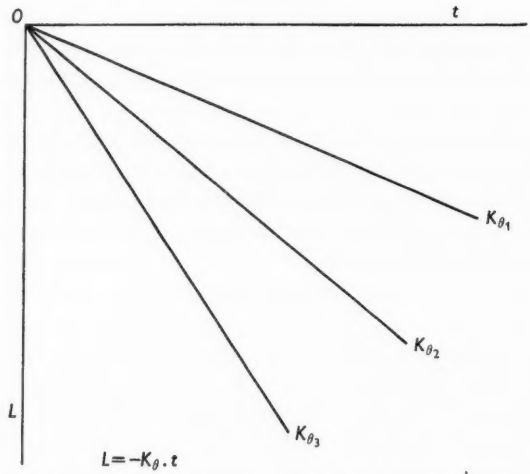


Fig. 1. Variation of $\log_{10}$ (proportion of surviving organisms) with time for temperatures $\theta_1$, $\theta_2$, $\theta_3$.

## CALCULATION OF THE LETHAL EFFECT OF A HOLDING SECTION

### *Theoretical discussion*

In a holding section as generally understood, the temperature can be considered constant at all times. The problem is therefore to determine the integrated lethal effect on a number of particles of liquid, different numbers of which pass through the holding section in different times. Suppose that the holding temperature is $\theta$. The thermal death-time characteristic of organisms at this temperature is, from Equation (1),

$$L=-K_\theta t.$$

The times for which organisms are subjected to the temperature $\theta$ are given by the distribution of flow times for the holding section, determined as described in the first paper of this series (1). This distribution shows the relationship between holding time, and the proportion of liquid which has been subjected to that holding time.

Fig. 2 shows the distribution of holding times for the plant considered in this series, this being a representation of Fig. 5 of the previous paper. The scale of ordinates is that

of nitrite concentration and is therefore arbitrary. The proportion of liquid passing at a given holding time bears some constant relation, however, to the nitrite concentration for that time. Suppose that the curve of proportion of liquid against time of flow through the holding tube, or holding time, is represented by the 'holding time function', $H(t)$.

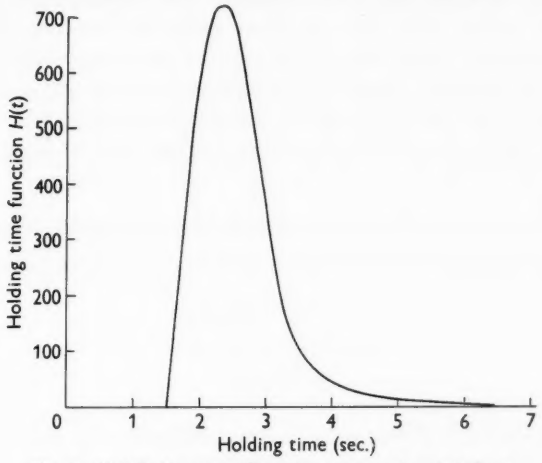


Fig. 2. Distribution of holding time for experimental plant.

If we consider a number of organisms all entering the holding tube together, then the number of organisms passing through the holding section in a small interval of time $dt$ about a mean time of travel $t$ is $H(t)dt$. This number of organisms has been subjected to a temperature $\theta$ for a time $t$.

If the proportion of surviving organisms is $R$, then

$$R = 10^{L}.$$

But from (1),

$$L = -K_\theta t,$$

therefore

$$R = 10^{-K_\theta t}.$$

Therefore of the $H(t)dt$ organisms subject to a holding time $t$, the number of survivors is

$$dN = 10^{-K_\theta t} H(t)dt. \tag{6}$$

If we consider all the organisms contained in the holding time distribution $H(t)$, the total number of survivors is given by the integral of (6) over the whole distribution. Therefore

$$\text{Number of survivors} = \int_0^\infty 10^{-K_\theta t} H(t)dt.$$

But the total number of incident organisms must be given by the integral of the 'holding time function' over the same range of holding times. Therefore

$$\text{Number of incident organisms} = \int_0^\infty H(t)dt.$$

Therefore for the total number of organisms, the proportion of survivors

$$R = \frac{\int_0^\infty 10^{-K_\theta t} H(t)dt}{\int_0^\infty H(t)dt}. \tag{7}$$

This fraction can be computed graphically for any given holding time function, $H(t)$, and value of the slope of the thermal death curve $K_\theta$. $H(t)$ is characteristic of the plant being considered, and must be determined experimentally. $K_\theta$ is the value for the organism of greatest importance at the temperature of operation. Therefore differences in type of organisms and in temperature of operation are both accounted for by the choice of a suitable value of $K_\theta$. This must be chosen either by direct experimental determination, or by reasonable extrapolation from other experimental evidence.

By computing the fraction of survivors for a known function $H(t)$ and a series of values of $K_\theta$, the result can be made general for one particular plant. The effect of different organism thermal resistances, or of temperature on one type of organism, can then be seen directly.

*Computation of the lethal effect for the experimental plant*

This involves the graphical determination of the ratio given in Equation (7):

$$R=\frac{\int_0^\infty 10^{-K_\theta t}H(t)\,dt}{\int_0^\infty H(t)\,dt}.$$

A typical computation is given for the function $H(t)$ shown in Fig. 2, and for $K_\theta=0{\cdot}5$. The steps of the computation are set out in Table 1.

Table 1. *Steps in computation of lethal effect for* $K_\theta=0{\cdot}5$ *and* $H(t)$ *as shown in Fig.* 2

| $t$ (sec.) | $H(t)$ | $10^{-0{\cdot}5t}$ | $10^{-0{\cdot}5t}H(t)$ |
|---|---|---|---|
| 1·65 | 95 | 0·1481 | 14·2 |
| 1·75 | 210 | 0·1332 | 27·9 |
| 1·85 | 360 | 0·1186 | 42·6 |
| 1·95 | 510 | 0·1058 | 53·9 |
| 2·05 | 630 | 0·0945 | 59·5 |
| 2·15 | 694 | 0·0836 | 58·1 |
| 2·25 | 713 | 0·0749 | 53·4 |
| 2·35 | 720 | 0·0667 | 48·0 |
| 2·45 | 718 | 0·0595 | 42·6 |
| 2·55 | 700 | 0·0531 | 37·2 |
| 2·65 | 630 | 0·0473 | 29·8 |
| 2·75 | 530 | 0·0422 | 22·4 |
| 2·95 | 325 | 0·0334 | 10·9 |
| 3·15 | 210 | 0·0266 | 5·6 |
| 3·35 | 136 | 0·0212 | 2·9 |
| 3·75 | 60 | 0·0133 | 0·8 |

$$\int_0^\infty 10^{-0{\cdot}5t}H(t)\,dt=55{\cdot}75.$$

$$\int_0^\infty H(t)\,dt=883.$$

$$\text{Proportion of survivors, } R=\frac{\int_0^\infty 10^{-0{\cdot}5t}H(t)\,dt}{\int_0^\infty H(t)\,dt}=6{\cdot}33\times10^{-2}.$$

The integral $\int_0^\infty H(t)\,dt$ is the total area under the curve of Fig. 2, which can be found either by planimeter or by counting squares. For one holding time function, this integral has a constant value.

The integral $\int_0^\infty 10^{-K_\theta t} H(t)\,dt$ is obtained by plotting the values of $10^{-K_\theta t} H(t)$ against time, followed by a graphical integration as above. This auxiliary curve may be termed the 'holding time survivor curve', and is shown for $K_\theta = 0{\cdot}5$ in Fig. 3. It may be considered to represent the probability of viable organisms leaving the holder for different times of passage.

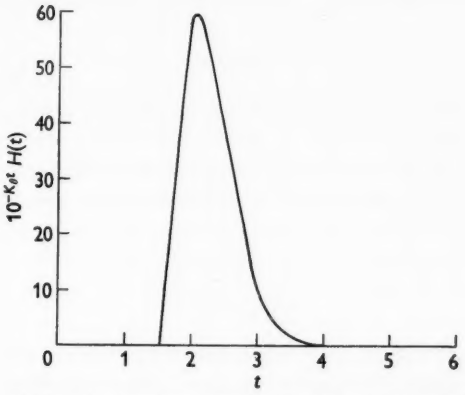


Fig. 3. Holding time survivor curve for $K_\theta = 0{\cdot}5$ and $H(t)$ as in Fig. 2.

$\int_0^\infty 10^{-K_\theta t} H(t)dt$ = area under curve = 55·75 units.

As a matter of interest, it can be seen from Figs. 2 and 3 that, while more organisms pass through the holder in about 2·3 sec. than any other time, the maximum number of surviving organisms passes at about 2 sec. As the value of $K_\theta$ increases, i.e. the rate of destruction of organisms increases, the time at which the maximum number of surviving organisms passes through the holder decreases. When $K_\theta$ is 4, the maximum probability of an organism surviving occurs at a flow time of about 1·6 sec.

Table 2 shows the variation of the proportion of surviving organisms, $R$, due to the holding section for values of $K_\theta$ of 0·5, 1·0 and 2·0.

Table 2. *Proportion of surviving organisms for the holding section, R, for H(t) as shown in Fig. 2 and different values of* $K_\theta$

| $K_\theta$ | $R$ |
|---|---|
| 0·5 | $6{\cdot}33 \times 10^{-2}$ |
| 1·0 | $4{\cdot}91 \times 10^{-3}$ |
| 2·0 | $4{\cdot}43 \times 10^{-5}$ |

## CALCULATION OF THE LETHAL EFFECT OF A HEATING SECTION

### *Theoretical discussion*

The calculation of lethal effect for the heating section is more involved than that for the holding section, since two independent sets of variables are involved. As in the previous section there is the distribution of flow times, but in addition there is the variation of temperature with distance through the plant. Different particles of liquid, as their mean

velocities differ, will be subjected to different time-temperature conditions, which can be represented by different time-temperature curves. In all of these the temperature scale will remain the same, but the time scale will vary.

Let us in the first place consider a case in which the liquid is all assumed to pass through the plant in the same time. This will correspond to the liquid all being subjected to the same time-temperature treatment, the total time being that required for the liquid to pass from one end to the other of the heating section. This time-temperature relation can be expressed as

$$\theta_t = f(t),$$

where $\theta_t$ has its maximum value $\theta$, the holding temperature, when $t$ equals the total time of travel through the heating section, $T$. At any point in the heat exchanger, the slope of the thermal death curve, $K$, has a value corresponding to the temperature at that point.

At any time $t$ on the equivalent time–temperature curve, where the temperature is $\theta_t$, we have from Equation (1)

$$L = -K_{\theta_t} t,$$

therefore

$$dL = -K_{\theta_t} dt.$$

If we integrate the lethal effects at all points in the heating section, we have for the logarithm of the proportion of surviving organisms for a flow time, $T$,

$$L_T = \int_0^T -K_{\theta_t} dt. \tag{8}$$

This integral can be determined graphically as in the previous section if the variation of $K_{\theta_t}$ with $t$ is known.

The variation of temperature with time is known from the experimental investigation of the plant. If the value of $K_\theta$ corresponding to the maximum temperature in the heating section, $\theta$, is known, the value of $K$ at any lower temperature can be determined by the relation of Equation (4), which can take the form

$$K_{\theta-1} = K_\theta / Q_1. \tag{9}$$

The variation of $K_{\theta_t}$ with $t$ can therefore be found from the temperature data, and can be used for the evaluation of the integral of Equation (8). The bacteriological data required are the value of $K_\theta$ at temperature $\theta$, which was used in the previous calculation for the holding section, and the $Q_1$ for the organisms at temperatures below the operating temperature.

In practice it is found that by far the greater part of the lethal effect occurs at temperatures within about 10° C. of the maximum, or holding, temperature. There is little inaccuracy involved in assuming and using a constant $Q_1$ over this temperature range. While the value of $K_\theta$ to be used should be the best estimate from the available experimental data of the value of $K$ at the holding temperature, the value of $Q_1$ should preferably refer to a temperature some 5° C. below the holding temperature. In fact the $Q_1$ corresponding to the holding temperature can be used without difficulty.

The method by which these principles are applied to evaluating the results for one particular plant will be set out in the succeeding section.

From the logarithm of the proportion of surviving organisms, the numerical proportion

of surviving organisms can be found. If $S_T$ is the proportion of surviving organisms for a flow time $T$,

$$S_T = 10^{L_T}. \tag{10}$$

It is now necessary to modify this result to take account of the experimentally determined distribution of the flow times in the heating section.

Since the effect of varying the time of flow through the plant is to extend the time scale in the integral of Equation (8) without having any effect on the other factors, it is possible to obtain the integral for any new value of flow time, $T$, by simple multiplication by the ratio of the times. If $L_T$ is the logarithm of the proportion of survivors for a flow time $T$, then for the same spacial distribution of temperature in the plant, the logarithm of the proportion of survivors for a flow time, $T_1$, is given by

$$L_{T_1} = L_T \frac{T_1}{T}. \tag{11}$$

It is therefore a simple matter to obtain from a detailed calculation for one flow time, the number of surviving organisms for any other flow time. The calculation for the integral result of the whole distribution then follows a similar course to that used earlier for the holding section. Suppose that the curve of proportion of liquid against time of flow through the heating section of the plant is represented by the 'heating time function', $I(T)$. Then the number of organisms passing through the heating section in a small interval of time $dT$ about a mean time of travel $T$ is $I(T)dT$. This number of organisms will have a proportion of survivors $S_T$ given by Equations (8) and (10).

The number of surviving organisms in this small element is therefore

$$dN = S_T . I(T) dT. \tag{12}$$

If we consider all the organisms contained in the heating time distribution $I(T)$, the total number of survivors is given by the integral of (12) over the whole distribution. Therefore

$$\text{number of survivors} = \int_0^\infty S_T . I(T)\, dT.$$

But the total number of organisms incident on the heating section is given by the integral of the heating time function over the same range of heating times. Therefore

$$\text{number of incident organisms} = \int_0^\infty I(T) dT.$$

Therefore for the total number of organisms, the proportion of survivors is

$$S = \frac{\int_0^\infty S_T . I(T) dT}{\int_0^\infty I(T) dT}. \tag{13}$$

These integrals can be determined graphically when $S_T$ is known for a series of times of flow, and this is possible by use of Equations (8) and (10). $I(T)$ is already known directly from experiment. As in the case of the holding section, the value of $S$ can be plotted as a function of $K_\theta$ at the temperature of operation, the value of $Q_1$ being constant. The process can be repeated for other values of $Q_1$ to cover the range likely to be met with for important organisms.

*Computation of the lethal effect for the experimental plant*

This has been carried out for values of $K_\theta$ of 0·5, 1·0 and 2·0 as for the holding section, and for values of $Q_1$ of 1·20, 1·25, 1·30, 1·35 and 1·40. These latter correspond to a range of $Q_{10}$ of from 6·2 to 29·0, which is expected to cover the values for resistant spores in practice.

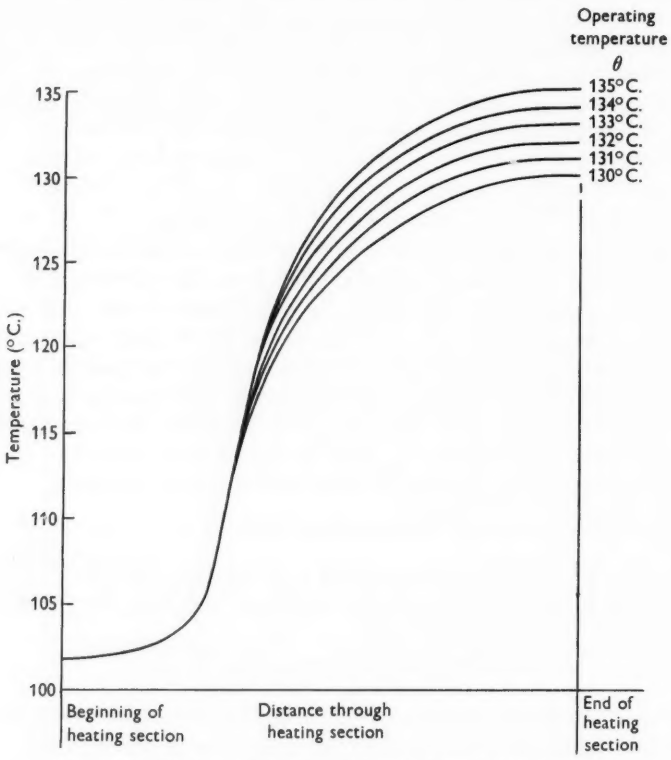


Fig. 4. Smoothed curves for temperature in steam heating section.

Typical experimental curves for the variation of temperature with position in the heating section of the plant considered in this series were given as Fig. 3 in the previous paper(1). While it is actually possible to deal with irregular temperature curves of this form, it is more convenient to deal with smoothed curves. Furthermore, smooth curves of a generally exponential form are those which would be expected in practice in the absence of complicating factors which, as discussed in the previous paper, are thought to have been present here. The irregular curves have therefore been smoothed for use in the calculations. From the results obtained on the plant, typified by the figure given, it is possible by slight adjustment of the curves to draw temperature distributions for the heating section of the plant for a series of final temperatures. These temperatures have been chosen at 1° C. intervals from 130° to 135° C., as being the range of chief interest in connexion with ultra-high-temperature heating on this plant. The set of smoothed temperature curves obtained is shown in Fig. 4.

A generalization is possible since it can be seen that, over the range of final temperatures of 130°–135° C., temperatures 1°, 2°, etc., below the final temperature occur in all the curves at very nearly the same points on the abscissae scale. This is true for temperatures at least 10° C. below the final temperature and hence for the range of temperatures giving the main part of the lethal action. It is therefore possible, for the range of final temperatures given, to combine the different curves into one curve. This relates temperature relative to the final temperature, to position in the plant, and is shown in Fig. 5.

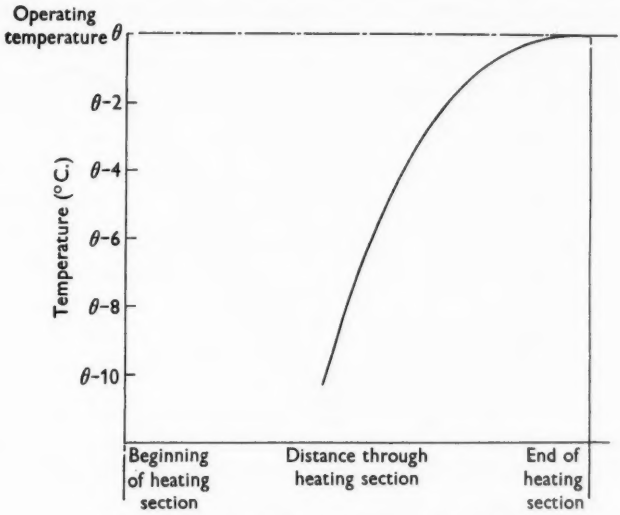


Fig. 5. Common heating curve representing Fig. 4.

A common curve of this kind can be shown to have some validity in theory, but it seems sufficient here that it represents closely the conditions for the group of curves in Fig. 4.

It is now possible to carry out the integration of Equation (8) above. It is convenient to give the total flow time $T$ for the purpose of this integration the hypothetical value of unit time, i.e. 1 sec. This enables the integral for any flow time to be determined by a single multiplication of the basic integral by the flow time in seconds. It is also convenient to plot the curve of $K_{\theta_t}$ against $t$ for $K_\theta = 1$ at the maximum temperature $\theta$. It is then possible to find the integral for unit flow time and any other value of $K_\theta$ by multiplication of the basic integral by the new value of $K_\theta$.

The curves of $K_{\theta_t}$ against time, for $K_\theta = 1$ and $T = 1$, and for the five values of $Q_1$ chosen, are shown in Fig. 6. These curves are derived from the temperature curve of Fig. 5 by the method described in the previous section using Equation (9). The value of the integral of Equation (8) is then given by the area under the curve for each of the values of $Q_1$. The values are given in Table 3. To be converted to the values for any other heating time, they must be multiplied by that heating time in seconds.

The flow-time distribution for the heating section for which the computation is made, $I(T)$, is shown in Fig. 7, which is a reproduction of Fig. 7 of the previous paper (1).

A typical computation for the integrated effect of the heating section is set out in Table 4 for the case in which $K_\theta = 0{\cdot}5$ and $Q_1 = 1{\cdot}30$.

The proportion of survivors under these conditions can be determined as for the holding section, by plotting from the values of $S_T.I(T)$ in the last column of Table 4 a heating time survivor curve analogous to that of Fig. 3 for the holding time. The integral of this survivor curve, divided by the integral of the distribution curve of Fig. 7, gives the proportion of surviving organisms under these conditions. This step will not be given in detail, since it follows exactly the procedure for the holding section. The proportion of survivors determined in this way for some different values of $K_\theta$ and $Q_1$ are set out in Table 5.

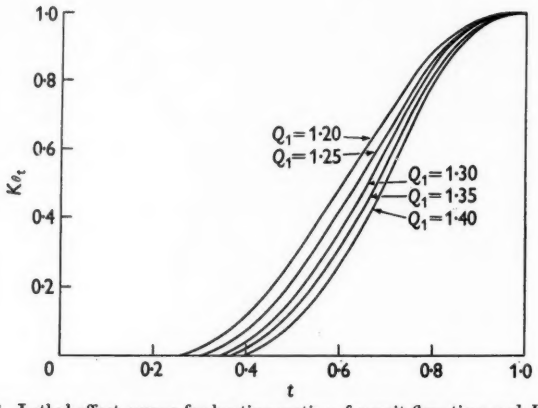


Fig. 6. Lethal effect curves for heating section, for unit flow time and $K_\theta = 1$.

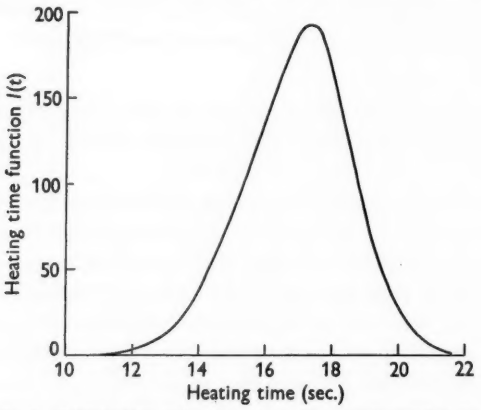


Fig. 7. Distribution of heating times for experimental plant.

Table 3. *Value of logarithm of proportion of surviving organisms for heating section*

(Hypothetical heating time = 1 sec. $K_\theta = 1$.)

| $Q_1$ | $L$ |
|---|---|
| 1·20 | −0·3976 |
| 1·25 | −0·3696 |
| 1·30 | −0·3496 |
| 1·35 | −0·3298 |
| 1·40 | −0·3100 |

Table 4. *Steps in computation of lethal effect for* $K_\theta = 0{\cdot}5$, $Q_1 = 1{\cdot}30$, *and* $I(T)$ *as shown in Fig.* 7

| $T$ (sec.) | $L_T$ | $S_T$ | $I(t)$ | $S_T.I(T)$ |
|---|---|---|---|---|
| 11 | $-1{\cdot}923$* | $1{\cdot}195 \times 10^{-2}$† | 1 | $1{\cdot}19 \times 10^{-2}$ |
| 12 | $-2{\cdot}095$ | $8{\cdot}03 \times 10^{-3}$ | 4 | $3{\cdot}21 \times 10^{-2}$ |
| 13 | $-2{\cdot}272$ | $5{\cdot}34 \times 10^{-3}$ | 14 | $7{\cdot}47 \times 10^{-2}$ |
| 14 | $-2{\cdot}447$ | $3{\cdot}56 \times 10^{-3}$ | 38 | $13{\cdot}54 \times 10^{-2}$ |
| 15 | $-2{\cdot}620$ | $2{\cdot}40 \times 10^{-3}$ | 80 | $19{\cdot}21 \times 10^{-2}$ |
| 16 | $-2{\cdot}795$ | $1{\cdot}605 \times 10^{-3}$ | 142 | $22{\cdot}78 \times 10^{-2}$ |
| 17 | $-2{\cdot}970$ | $1{\cdot}07 \times 10^{-3}$ | 191 | $20{\cdot}50 \times 10^{-2}$ |
| 18 | $-3{\cdot}148$ | $7{\cdot}11 \times 10^{-4}$ | 160 | $11{\cdot}39 \times 10^{-2}$ |
| 19 | $-3{\cdot}322$ | $4{\cdot}76 \times 10^{-4}$ | 80 | $3{\cdot}01 \times 10^{-2}$ |
| 20 | $-3{\cdot}496$ | $3{\cdot}19 \times 10^{-4}$ | 30 | $0{\cdot}96 \times 10^{-2}$ |

* Integral of Equation (8) for hypothetical flow time of 1 sec., $K_\theta = 1{\cdot}0$ and $Q_1 = 1{\cdot}30$ is $-0{\cdot}3496$ (from Table 3). Therefore integral for hypothetical flow time of 1 sec., $K_\theta = 0{\cdot}5$ and $Q_1 = 1{\cdot}30$ is

$$-0{\cdot}3496 \times 0{\cdot}5/1{\cdot}0 = -0{\cdot}1748.$$

Therefore integral for flow time of 11 sec. $= L_T = -0{\cdot}1748 \times 11 = -1{\cdot}923$.

† $S_T = 10^{L_T}$ from Equation (10).

$$\int_0^\infty S_T.I(T)dT = 1{\cdot}033.$$

$$\int_0^\infty I(T)dT = 752.$$

$$\text{Proportion of survivors, } S = \frac{\int_0^\infty S_T.I(T)dT}{\int_0^\infty I(T)dT} = 1{\cdot}37 \times 10^{-3}.$$

Table 5. *Proportion of surviving organisms for the heating section,* $S$, *for different values of* $K_\theta$ *and* $Q_1$

| $K_\theta$ | $Q_1$ | $S$ |
|---|---|---|
| 0·5 | 1·20 | $5{\cdot}81 \times 10^{-4}$ |
| 1·0 | 1·20 | $6{\cdot}73 \times 10^{-7}$ |
| 2·0 | 1·20 | $5{\cdot}75 \times 10^{-12}$ |
| 2·0 | 1·25 | $2{\cdot}84 \times 10^{-11}$ |
| 0·5 | 1·30 | $1{\cdot}37 \times 10^{-3}$ |
| 1·0 | 1·30 | $3{\cdot}08 \times 10^{-6}$ |
| 2·0 | 1·30 | $9{\cdot}05 \times 10^{-11}$ |
| 2·0 | 1·35 | $2{\cdot}77 \times 10^{-10}$ |
| 1·0 | 1·40 | $1{\cdot}20 \times 10^{-5}$ |
| 2·0 | 1·40 | $7{\cdot}95 \times 10^{-10}$ |

### TOTAL LETHAL EFFECT OF EXPERIMENTAL PLANT

The rate of cooling of liquid in the plant is so rapid immediately after the holding section when the regenerator is reached, that only the heating and holding sections contribute to the overall lethal effect. In Fig. 8, the proportion of survivors is plotted logarithmically as a function of $K_\theta$ for the holding section (see Table 2) and for the heating section (see Table 5). The over-all proportion of survivors is the product of the two components, and is also given in Fig. 8.

From this curve, it is possible to predict the number of organisms which will survive treatment in the plant, provided that the thermal death of the organisms is logarithmic and has a slope of $K_\theta$ at the operating temperature and a $Q_1$ of any value between 1·20 and 1·40 about the operating temperature. Expression of the results in terms of $K_\theta$ means that any organism can be considered at any operating temperature within about 5° C. of

the nominal, merely by using the appropriate value of $K_\theta$. Variation in $Q_1$ gives comparatively little variation in the over-all lethal effect, but since the $Q_1$ will normally be found during the determination of $K_\theta$ at the operating temperature it can be easily taken into account if necessary.

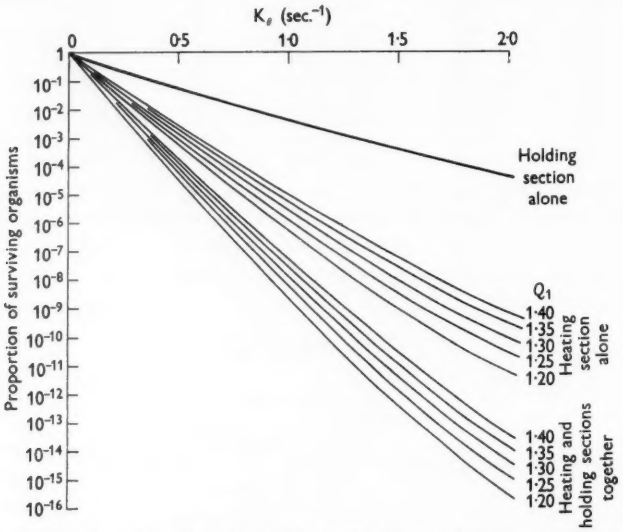


Fig. 8. Curves showing predicted bactericidal effect due to whole experimental plant.

It can be seen from the curves that, in this particular plant, the holding section has comparatively little lethal effect compared with the heating section. The holding time would have to be increased more than twice for the holding section to have any important bactericidal effect. If an increase in lethal effect is required, it can be obtained much more readily by an increase in temperature. This can be seen from the fact that the additional lethal effect due to the holding section could be obtained from the heating section alone merely by increasing $K_\theta$ by a factor of 1·5. Assuming a typical value of $Q_1$ for the organism of greatest importance, this corresponds to an increase in operating temperature of about 1·5° C.

## APPENDIX

### *The application of the method to concave-upwards non-logarithmic thermal death curves*

It is found that, with many resistant spore-forming organisms of importance in milk sterilization, the thermal death of the spores is not logarithmic. If the logarithm of the proportion of surviving spores is plotted as a function of time for a certain lethal temperature, the curve has a form similar to that of Fig. 9. It is clear that any assumption of a straight line will give serious errors in estimating the lethal effect if the thermal death curve is actually of this type.

However, it is possible to represent a curved line of this kind by a set of straight thermal death lines, each representing a population with a certain value of $K_\theta$ and having an initial concentration defined by the intercept of the line on the $L$ axis. The thermal death curve of the whole population is then the sum of those of its component parts.

The exact number of component straight lines is a matter of convenience and accuracy of fit. It is best to make the intercepts on the $L$ axis whole numbers, and then to draw the lines tangential to the curve to be matched. The number of lines should be kept to a minimum, and not more than three should give a reasonable fit to any experimental curve.

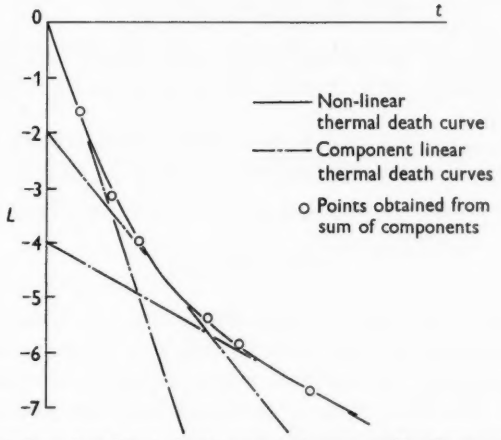


Fig. 9. Typical non-logarithmic death curve, and corresponding linear components.

For example, for the curve shown three component straight lines have been used, passing through $L=0$, $-2$ and $-4$ and tangential to the curve. Points obtained from the sum of the individual lines have been shown to indicate the accuracy of fit.

For calculation and reference to a graph such as Fig. 8, each population represented by a straight line can be considered separately, having its own value of $K_\theta$ at the operating temperature given by the slope of the line. For each population, a proportion of survivors can therefore be obtained. This must then be multiplied by the corresponding relative initial population, given by the intercept on the $L$ axis, to give a corrected proportion of survivors. The sum of these values for all the component populations is the over-all proportion of survivors for the curved thermal death line.

The fact that the sum of the separate populations gives a proportion greater than unity at zero time can be neglected since the error is so slight, e.g. a factor of 1·0101 in the example given.

I would like to thank Dr L. F. L. Clegg, Dr C. C. Thiel, Mr J. G. Franklin and Mr D. J. Williams for many useful discussions on this subject; Mr J. Hutchinson for checking the manuscript; and Mr L. D. R. Turner for help with the computations.

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# 722. AN ANALYSIS OF THE PERFORMANCE OF AN ULTRA-HIGH-TEMPERATURE MILK STERILIZING PLANT

## III. COMPARISON OF EXPERIMENTAL AND CALCULATED SPORICIDAL EFFECTS FOR A STRAIN OF *BACILLUS SUBTILIS*

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(With 4 Figures)

The previous papers of this series (1,2) have shown how the bactericidal efficiency of a flow-type heat exchanger may be predicted from physical measurements, and have given the necessary calculations for a small plate-type ultra-high-temperature sterilizing plant made by the A.P.V. Co. The purpose of this paper is to show the degree of agreement between practice and prediction from theory.

The bacteriological aspects of this work have already been partly described (3), and will be detailed more fully later (4,5). Only the results will be given here, with such details as are necessary.

### SPORICIDAL EFFICIENCY OF THE PLANT

The test organism used was a strain of *Bacillus subtilis* 786 isolated from bulk tanker milk. A 20 gal. quantity of whole milk was inoculated with a spore suspension of this organism to give a concentration of *c.* $5 \times 10^6$ spores/ml.

This volume of milk was treated in the sterilizing plant, with the minimum holding time, and with the steam pressure controlled by hand to give a constant treatment temperature. 2–3 l. of treated milk was collected in a sterile 10 l. aspirating jar fitted with a hooded outlet. The inoculated milk was also sampled at the same time.

Colony and dilution counts were made on the inoculated milk as a control after heating at 100° C. for 30 min. This has the effect of killing vegetative contaminants and of heat activating the spores. Dilution counts in litmus milk gave results similar to the colony counts.

Dilution counts were made on the treated milk by inoculating $1 \times 500$ ml., $5 \times 100$ ml., $5 \times 10$ ml., $5 \times 1$ ml. quantities, and so on as required. Whole milk which had been sterilized in the plant at an operating temperature of 135° C. and aseptically collected was used where necessary as the dilution medium.

Plates were incubated at 37° C. for 2 days, and dilution tubes at 37° C. for 1 week. As the test organisms produced no marked visual change in the ultra-high-temperature treated milk, all the samples in this medium were examined microscopically, and confirmation obtained by streak culture where necessary. The results of the dilution counts were obtained from McCrady's probability tables (6).

The results are given in Table 1.

Table 1. *The effectiveness of the heat treatment plant at different operating temperatures, for* Bacillus subtilis 786 *spores in milk*

| Operating temp. of plant (° C.) | *B. subtilis* spores/ml. in milk: Unheated (colony count) | Heated (dilution count) | Mean proportion of survivors |
|---|---|---|---|
| 130·5 | $7{\cdot}15 \times 10^6$ | 0·9 | $1{\cdot}07 \times 10^{-7}$ |
| | $6{\cdot}9 \times 10^6$ | 0·6 | |
| 132·0 | $4{\cdot}75 \times 10^6$ | 0·2 | $3{\cdot}03 \times 10^{-8}$ |
| | $4{\cdot}8 \times 10^6$ | 0·09 | |
| 133·5 | $4{\cdot}7 \times 10^6$ | 0·001 | $1{\cdot}67 \times 10^{-10}$ |
| | $7{\cdot}25 \times 10^6$ | 0·001 | |
| 135·0 | $4{\cdot}85 \times 10^6$ | <0·0004 | $<6{\cdot}1 \times 10^{-11}$ |
| | $8{\cdot}2 \times 10^6$ | <0·0004 | |

## LABORATORY THERMAL DEATH DATA

A suspension of spores of *B. subtilis* 786 was prepared having a concentration of *c.* $10^8$/ml. Thermal destruction curves were obtained for these spores at temperatures of 112·5, 115·0, 117·5 and 120·0° C. using the capillary tube method of Franklin, Williams & Clegg (5) based on that of Stern & Proctor (7). In the earlier experiments survivors were estimated by both colony and dilution counts, but later, because of the close agreement obtained between the two, colony counts only were used.

Whole milk sterilized in the plant at 135° C. was used for the preparation of the suspension, for the serial dilutions, and as the recovery medium in the dilution counts. Plates and dilution tubes were incubated and tested as above.

## APPLICATION OF THE LABORATORY DATA

### *Determination of* $Q_{10}$

From the laboratory experiments three sets of data were available, each relating the proportion of surviving spores to time of heating for a series of treatment temperatures. Because of the variability of the spore behaviour encountered in the laboratory experiments, it is necessary to take into account all the data to give the best estimate of $Q_{10}$.

The determination of $Q_{10}$ makes use of Arrhenius's equation, discussed briefly in the previous paper (2). It follows from this equation that, if a fixed survivor ratio is considered in a set of thermal death curves for different temperatures, then the logarithm of the time to reach that survivor ratio at each temperature is linearly related to the inverse of the absolute temperature.

For example, one set of laboratory results is given in Fig. 1. If a survivor ratio of $10^{-3}$ is considered, then the four points $P_1$, $P_2$, $P_3$, $P_4$ should be linearly related in the above way. The ordinates of the relation are $\log_{10} 1{\cdot}0 = 0$, $\log_{10} 2{\cdot}75 = 0{\cdot}44$, $\log_{10} 4{\cdot}75 = 0{\cdot}68$, and $\log_{10} 14{\cdot}7 = 1{\cdot}17$. The corresponding abscissae are

$$1/(120+273) = 2{\cdot}544 \times 10^{-3}, \qquad 1/(117{\cdot}5+273) = 2{\cdot}561 \times 10^{-3},$$
$$1/(115+273) = 2{\cdot}577 \times 10^{-3} \quad \text{and} \quad 1/(112{\cdot}5+273) = 2{\cdot}594 \times 10^{-3}.$$

From the linear relation, the $Q_{10}$ can be found.

The values for the three sets of laboratory results, at the three survivor ratios $10^{-3}$, $10^{-4}$ and $10^{-5}$, are given in Fig. 2. The best-fitting straight lines for each survivor ratio

have been drawn by inspection. For a change in abscissae of $2{\cdot}577 \times 10^{-3}$ to $2{\cdot}544 \times 10^{-3}$, corresponding to the change of 5° C. from 115° to 120° C., the ordinate changes by 0·75 for each of the straight lines. The change corresponding to 10° C. is therefore very nearly $0{\cdot}75 \times 2 = 1{\cdot}50$. This gives $Q_{10}$ as $10^{1{\cdot}50} = 31{\cdot}7$, and $Q_1$ as $31{\cdot}7^{0{\cdot}1} = 1{\cdot}41$.

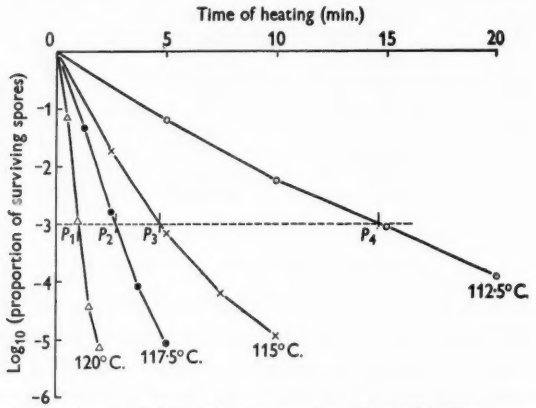


Fig. 1. One set of thermal death curves for *B. subtilis* 786 spores.

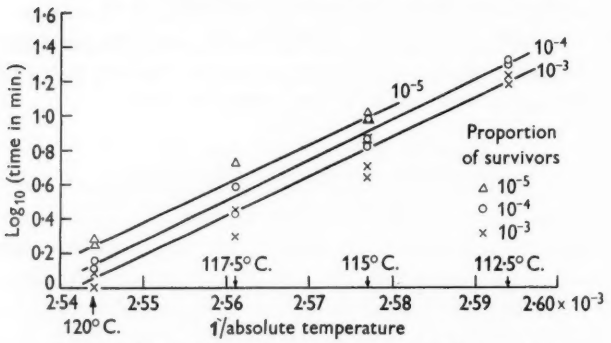


Fig. 2. Relation between thermal death rate and temperature.

*Determination of $K_\theta$ and the proportion of surviving spores*

Four sets of laboratory results were obtained for the thermal death of *B. subtilis* 786 spores at 115° C. These are all given in Fig. 3. There is some variation between experiments, and a considerable curvature in the thermal death curve at low survivor ratios. The mean thermal death curve can be represented by two linear populations as shown. The first has an initial relative concentration of unity and a $K_\theta$ at 115° C. of 0·525 min.$^{-1}$, or 0·0087 sec.$^{-1}$. The second has an initial concentration of $3{\cdot}16 \times 10^{-4}$ and a $K_\theta$ at 115° C. of 0·119 min.$^{-1}$ or 0·00198 sec.$^{-1}$.

The corresponding values of $K_\theta$ at higher temperatures can be obtained to a sufficient degree of accuracy, considering the other approximations which have been necessary, by a simple application of the $Q_1$ value, e.g.

$$K_{\theta_2} = K_{\theta_1} . Q_1^{\theta_2 - \theta_1},$$

where

$$Q_1 = 1{\cdot}41.$$

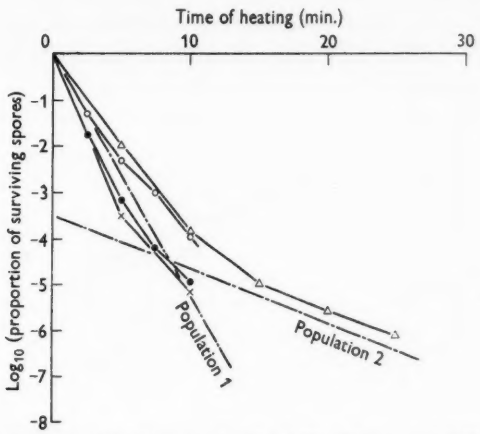


Fig. 3. Thermal death curves for *B. subtilis* 786 spores at 115° C.

Table 2. *Values of $K_\theta$ for two component spore populations at operating temperatures of* 129°–134° C.

| $\theta$ (° C.) | $K_\theta$ for first population (sec.$^{-1}$) | $K_\theta$ for second population (sec.$^{-1}$) |
|---|---|---|
| 129 | 1·09 | 0·25 |
| 130 | 1·55 | 0·35 |
| 131 | 2·18 | 0·495 |
| 132 | 3·05 | 0·695 |
| 133 | 4·35 | 0·99 |
| 134 | — | 1·39 |

Table 3. *Proportion of surviving spores for two component spore populations, obtained from Fig.* 8 *of the previous paper*

| $\theta$ (° C.) | Proportion for first population | Proportion for second population | Over-all proportion for second population (allowing for initial concentration) |
|---|---|---|---|
| 129 | $1 \times 10^{-8}$ | $1{\cdot}3 \times 10^{-2}$ | $4{\cdot}1 \times 10^{-6}$ |
| 130 | — | $2{\cdot}5 \times 10^{-3}$ | $7{\cdot}9 \times 10^{-7}$ |
| 131 | — | $2{\cdot}3 \times 10^{-4}$ | $7{\cdot}3 \times 10^{-8}$ |
| 132 | — | $8{\cdot}0 \times 10^{-6}$ | $2{\cdot}5 \times 10^{-9}$ |
| 133 | — | $6{\cdot}3 \times 10^{-8}$ | $2{\cdot}0 \times 10^{-11}$ |
| 134 | — | $1{\cdot}5 \times 10^{-10}$ | $4{\cdot}7 \times 10^{-14}$ |

The values of $K_\theta$ for the two populations at temperatures of 129° to 134° C. are given in Table 2. The corresponding values for the proportion of surviving spores, obtained from Fig. 8 of the previous paper of this series (2) for a $Q_1$ of 1·41, are given in Table 3. To give the over-all number of survivors in the second population, the proportion of survivors has to be multiplied by the initial concentration, $3{\cdot}16 \times 10^{-4}$. From the results for the lowest temperature, it is clear that the survivors from the first population can be neglected compared with those from the second. The results for the first population are therefore not given for the higher temperatures.

A comparison between the proportion of survivors found experimentally (from Table 1) and the proportion obtained by calculation (from Table 3) is given in Fig. 4.

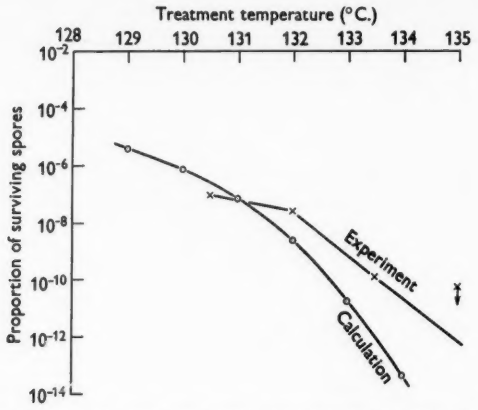


Fig. 4. Comparison of experimental and calculated lethal effects.

## DISCUSSION

There is good agreement between the calculated and experimental results, not only in the magnitudes of the sporicidal effects but also in the form of their variationw ith temperature. It seems that the method of calculation used is satisfactory for determining the performance of a plant.

At the same time, it must be recognized that the accuracy of the calculation depends entirely on the accuracy with which thermal death curves can be obtained in the laboratory at very low survival ratios. The results given in Fig. 3 show how difficult it is to obtain reproducible results on a spore suspension. The good agreement of experiment and calculation would have been less good if any one of the four separate sets of results had been used rather than the mean. Similarly, the agreement would have been poor if a linear thermal death curve had been assumed, with thermal death data obtained for short heating times extrapolated to longer times and smaller numbers of survivors on a linear basis.

It is probable that the thermal death curve of *B. subtilis* 786 continues as a curve to longer times and lower survival levels than are given in Fig. 3. The assumption of two linear component populations then underestimates the survival ratio to be expected. This may account for the increasing divergence between experimental and calculated results for survival ratios below about $10^{-8}$, i.e. at levels beyond those which can be obtained conveniently in laboratory thermal death determinations.

These results refer to milk containing one strain of spore-forming organism only, and show that the theory is adequate in that case. However, bacteriological considerations again make it unlikely that the performance of a plant under particular commercial conditions could be determined, since the plant would then work with a mixed spore population, each type of spore being present in a different proportion and having different thermal death characteristics. Such a system is too complex for theoretical analysis. The theory may be useful as a method for comparing the probable performances of different plants. For this purpose, a single spore population having a linear thermal death curve and suitable assumed thermal death characteristics can be considered. All the bacterio-

logical difficulties are then removed by the use of an assumed ideal population. A plant which is superior to another in such a case will almost certainly be superior under practical operating conditions. There may be cases in which such a comparison of bacteriological performance using only physical data from the plant will be simpler and more convenient than a direct bacteriological determination.

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# REVIEWS OF THE PROGRESS OF DAIRY SCIENCE

## SECTION D. NUTRITIVE VALUE OF MILK AND MILK PRODUCTS

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## PART 1

### INTRODUCTION

This section reviews a selection of papers which have reached New Zealand since the last review (1) was completed in January 1956. It covers some general aspects of the nutritive value of milk for man, and then deals more specifically with the nutritive value of milk fat and the associated fat-soluble vitamins. This review, like the last, represents a drastic departure, directed by *Journal* policy, from the comprehensive earlier reviews of Kon & Henry; limitations of space have again necessitated a much more selective approach, and the reviewer regrets the very large number of valuable contributions to which not even passing reference can be made.

It would seem that the period under review has again not been marked by any very significant advances in knowledge. Of the literature perused during the preparation of this review, however, a most striking feature has been the number of reports relating to possible associations between fat and related materials in the diet and the incidence of coronary diseases. Although in the meantime the bulk of this work is outside the scope of this review, and will remain so until the causes of atherosclerosis are more clearly defined, it is a field which may become of vital importance to nutritionists and others interested in maintaining and increasing the present consumption of milk and its products. Already the scant information is being exploited to promote the sales of other, non-animal products. In the United States, at least, the importance to the dairy industry of the frequent reference to milk fat as a factor in heart disease is clearly recognized, and both the National Dairy Council (2) and the American Dairy Association (3) are actively engaged in releasing to the public reliable information on this controversial subject and in telling a positive story of the nutritive value of milk and milk products. Appraisals of the present

situation can be gained from several recent reviews on various aspects of the subject (4,5,6,7,8). The last reference relates to a recent symposium on fats in nutrition and health. In summarizing this Goldsmith (8) draws attention to the hypotheses and few established facts on which so much speculation and opinion in the lay press has been based, but ends on the note that '. . . in spite of the paucity of current knowledge, there is sufficient evidence which suggests that dietary fat and serum lipid levels may be implicated in the development of atherosclerosis and coronary heart disease to justify intensive exploration of all facets of this and related fields. Information obtained from this research should prove valuable in understanding the role of fats in human nutrition, irrespective of findings relative to the atherosclerosis problem'.

## NUTRITIVE VALUE OF MILK FOR MAN

World milk production showed signs of levelling off during 1955 (9), when production in eighteen primary milk-producing countries increased by only 0·6% over 1954 figures. The post-war upward trend was, however, resumed in 1956 (10), when an increase of 2% over 1955 figures was recorded. Considering the 2-year period as a whole, increases were recorded in production of both butter and cheese (11), so that the over-all pattern of world milk utilization has remained relatively unchanged.

The XIVth International Dairy Congress was held in Rome during the period covered by this review, and as usual the reports (12) contain much information about the nutritive value of milk. A number of individual papers are referred to later.

### (*a*) *Adult nutrition*

Several workers, particularly contributors to the Dairy Congress, have dealt with the position of milk and its products in the diets of various countries and with ways of increasing consumption, and have again emphasized price in relation to income as a limiting factor. Khurody (13) has produced an interesting comparison of milk consumption and purchasing power in twenty-two countries. As income increases there are also changes in milk utilization patterns resulting in a greater quantity of milk being purchased for direct consumption (14,15). At the same time economic considerations cannot be taken too far; two recent surveys (16,17) showed, as the main reason for suboptimal milk consumption, not price but a plain and simple dislike of its taste.

Kay, in an article on the future of quality milk (18), draws attention to the fact that although the keeping quality and hygienic quality of milk have improved in recent years, nutritional quality in terms of fat and solids-not-fat has been declining. The same problem is being encountered in parts of New Zealand where a recent symposium suggested fortifying, at the expense of the producer, poor-quality milk with dried skim-milk powder to bring it up to an accepted nutritional (and legal) standard (19).

The nutritive value of milk has been reviewed by several workers (20,21,22). An evaluation of the nutrients of milk and its products in terms which can be presented to, and understood by, the average consumer is given in (23). In feeding trials with children, Natvig (24) confirms the generally accepted view that there is no evidence of any real differences between the values of raw and pasteurized milks. Wagner (25) discusses the changes in milk proteins and the vitamin losses during pasteurization, sterilization and uperization, and reports that rats fed raw milk gave the highest weight gains. On the other hand, an extensive investigation by de Groot & Engel (26) failed to establish any significant differences between the nutritive values of raw, pasteurized and sterilized milks. Mancini (27) also concludes that sterilized and pasteurized milks are almost identical in nutritive value. He also emphasizes the point that even if sterilized milk should prove to be nutritionally inferior, it is still to be preferred under certain conditions owing to its better keeping quality.

Radiation sterilization of foods, including milk products, is attracting considerable attention. Metta & Johnson (28) found that irradiation decreased the biological value of proteins in evaporated milk, while Becker *et al.* (29) report impaired reproductive performance of rats fed irradiated

milk fat. Summarizing the present status of radiation sterilization of foods, Wasserman (30) considers that, as with most foods, destruction of micronutrients in milk is the main problem. He reports that sterilizing doses of gamma radiations destroy large percentages of vitamin A, ascorbic acid and tocopherols, and concludes that much further work is necessary before radiation sterilization of dairy products is possible commercially.

Turning to indigenous products Kopytine (31) discusses the nutritive value of Kherân, a fermented milk of the Siberian Tartars.

Some information on the nutritive value of cheese (32) and of butter (33) is given by Randoin & Causeret.

The production at the N.I.R.D., in response to a request from a shipping company, of deep-frozen milk and cream which will store satisfactorily for at least 12 months below 10° F. and will reconstitute to first-class pasteurized milk (34), will be welcomed by sea-travellers and may well lead to the increased availability and consumption of milk in difficult areas where cost is not a determining factor.

(*b*) *Milk in infant nutrition*

The period under review has again produced a very extensive literature on the value of different types of milk in infant feeding. Much of the work has related to comparisons of the nutritive value of human milk and suitably modified milk of other mammals. However, this field still remains in a most unsatisfactory and confused state, the conflicting results being attributable largely to the different techniques employed, some methods not necessarily measuring nutritive value and others being susceptible to factors entirely unrelated to the diets fed. As examples of the techniques employed and the results obtained, Gross & Moses (35), working on weight gains alone, Berger, Wavre & Gilardi (36) on the basis of growth curves, similarity of faecal flora and resistance to infection, and Guilbert, Baker & Barness (37) measuring fat retention and faecal pH and flora, could detect no significant differences between groups of infants fed human milk and various preparations based on modified cows milk.

Millis (38), also using weight gains, found varying results depending on the duration of the experiment and the particular groups chosen. Barness, Baker, Guilbert, Torres & György (39) and Zoula (40) found better nitrogen retention with human milk, and Mannheimer (41) reports a higher mortality rate in artificially fed infants, although he draws attention to the effect of family income on his results. *In vitro* techniques for studying peptic-tryptic breakdown of the proteins and fat in various milks have been employed by other workers (42, 43).

In general, recent work would seem to support the previous conclusions (1) that a number of artificial milks are, for all practical purposes, in no way nutritionally inferior to human milk. With the increasing incidence of artificial feeding (e.g. 44) there would seem to be a real need for the wider dissemination of reliable information on the nutritional value of these artificial milks.

Attention has been drawn to the possibilities of tocopherol (45) or vitamin $B_6$ (46) deficiencies in particular milk preparations used in infant feeding.

There are a number of reports on aspects of bifidus activity and intestinal bactobacilli (e.g. (47, 48, 49, 50), the latter reference referring to two United States patents covering the addition of bifidus-active substances to infant foods.

## MILK FAT

Useful information on the milk fat of several species is given in the 3rd edition of Hilditch's text on the chemical constitution of natural fats (51). A review of the chemistry of milk fat by Jack & Smith (52) covers much of interest to nutritionists, while Shorland & Hansen (53) have reviewed the composition, including minor nutrients, of New Zealand milk fat.

Reports continue to appear on the nutritive value of milk fat relative to margarine and other fats. Of eighteen natural fats tested, Thomasson (54) found that milk fat was the most rapidly absorbed. Growth action was found to depend on absorption rate, and the same worker (55) found that rats fed milk fat grew more rapidly, lived a shorter time and showed greater incidence

of pathological changes than those fed the same level of rapeseed oil. Investigating the absorption of various natural fats including milk fat, Calloway, Kurtz, McMullen & Thomas (56) found no relationship between digestibility and physical properties or chemical constitution. Hydrogenated fats, on the other hand, showed the expected variation in digestibility with chain length. The digestibility of natural milk fat was 98% and that of hydrogenated milk fat 61%. Rat-feeding experiments by Kehar, Krishnan, Ray, Kumar & Pal (57) disclosed only very slight differences in digestibility between various animal and vegetable fats. Dryden and co-workers, in experiments extending over several generations, found no significant differences in the reproductive or lactational performances of rats fed milk fat, margarine (58) or hydrogenated vegetable oil (59). In other experiments (60) the slight differences found between milk fat and maize oil diets depended on the level of fat fed. This latter paper also quotes previously unpublished findings of Carey and co-workers, further exemplifying the importance of the carbohydrate fraction of the diet on fat utilization. Lactose–milk fat diets yielded greater body weight gains than lactose–maize oil diets, but identical weight gains were achieved when the lactose was replaced by dextrin. Schweigart (61) has reviewed a number of papers which support his view that butter is superior to other fats, especially margarine.

The relationship between animal fats and atherosclerosis has already been referred to in general terms. Of the range of papers published in this field, a number seem relevant to this review since they appear to specifically implicate milk fat in contributing to conditions which may bear relationships to heart disease. Thus MacLagan & Billimoria (62) conclude that it is the phospholipid content of dairy products which is responsible for their greater effect on blood coagulation relative to vegetable fats. Ingestion of milk fat by humans produces a marked rise in blood cholesterol levels, whereas maize oil produces the opposite effect (63, 64). The factor in milk fat is associated with the more volatile fractions of the fat (65) and Bronte-Stewart, Antonis, Eales & Brock (66) suggest that the effect on blood cholesterol level is associated with the degree of saturation of the fatty acids fed. In the absence of more precise information one may only speculate on the significance of these findings.

## FAT-SOLUBLE VITAMINS

### (*a*) *Vitamin A and carotene*

A number of reports have appeared on the estimation of vitamin A and carotene. Moore's comprehensive text, *Vitamin A* (67), contains an excellent section on methods of estimation, as well as providing much general information on vitamin A, its provitamins and congeners. Booth (68) has also published a valuable text on the estimation of carotene in biological materials. A method for the direct extraction and estimation of vitamin A in milk is reported by Kadaba & Herreid (69), while a method of estimating vitamin A and carotene based on plate chromatography is described by Lagoni & Wortmann (70). Assay methods are also described in (71) and (72), the former reference describing methods currently in use at the N.I.R.D.

Levels of vitamin A and carotene, and seasonal variations in these levels, have been reported for milk and milk fat produced in a number of areas; these include Australia and Denmark (73), New Zealand (73, 74, 75, 76), two districts in Czechoslovakia (77), the Central Yakutz district of Russia (78), Upper Bavaria (79), Latvia (80), the province of Manitoba in Canada (81), the Sao Paulo province of Brazil (82) and India (83, 84, 85). The results are substantially in agreement with those reported previously for similar areas and the seasonal variations can, in most cases, be associated with periods of stall and pasture feeding. The Indian workers (83, 84) found significantly higher vitamin A levels during the monsoon seasons.

With good quality pasture available throughout the year, seasonal variations in vitamin A and carotene levels of the type reported for New Zealand milk fats are unexpected and difficult to explain. The changes have been shown to be seasonal in nature and almost entirely independent of lactational influences (74). Clearly, factors other than the carotene content of the pasture must influence the vitamin A potency of the milk fat and aspects of the problem have been investigated by McGillivray and co-workers (75, 76, 86, 87, 88, 89, 90). Variations in milk fat

vitamin A and carotene reflect seasonal changes in the blood levels of these constituents, although the carotene content of the pasture remains relatively constant throughout the year (75, 76). Botanical composition of the pastures and environmental factors would appear to be implicated in the seasonal variations (86, 89). The influence of pasture in general on the vitamin A and carotene content of cows' milk has been reviewed by McGillivray & Thompson (91).

An unusual investigation, but one of some nutritional importance, has been into the effects of small oral doses of paraffin oil, found satisfactory in New Zealand as a prophylaxis against bloat, on the levels of vitamin A and carotene in milk fat (92, 93). Recommended dosage levels of the oil have been found to produce a marked effect, particularly on the carotene content of the milk fat which is reduced by about 50%. Such a reduction in carotene content, and hence in colour, is of particular interest to the butter-factory operator since groups in Great Britain and the majority of consumers in Europe prefer a pale butter. The nutritional undesirability of such a reduction is, of course, obvious, although it is pointed out (93) that the total vitamin A potency of the fat from treated animals would not be below about 40 i.u./g., a level still high relative to milk fat produced in most other parts of the world.

It is somewhat paradoxical that while there is interest in New Zealand in reducing the natural carotene content of butter, increasing use is being made elsewhere of synthetic carotene as a butter colouring agent. This readily available and relatively cheap form of the pigment, used at levels of up to about 8 $\mu$g./g. fat, has no deleterious effects on the butter or its keeping qualities (e.g. (94, 95, 96)).

Further investigating the effect of ground soybean on the level of vitamin A and carotene in the milk fat of cows, Monroe, Hibbs & Washburn (97) found no evidence of destruction of carotene or interference with its absorption or conversion to vitamin A, provided ample good roughage was also available. Hjarde & Larsen (98), in experiments with fresh lucerne and lucerne silage, have further exemplified the influence of factors other than carotene intake on the vitamin A potency of milk fat. The dependence of animals on dietary fat-soluble vitamins for their secretion in the milk is also further demonstrated in (99). These, and the transitory effects on milk fat levels of single doses of vitamin A reported in (100) and also in (87), emphasize the immediate dietary origin of the bulk of milk fat vitamin A.

Randoin (101) investigated the changes in the vitamin A content of Gruyère cheese at different stages of processing. Appreciable losses were found to occur during the heating of the curd and during the first month. The preparation of Yoghurt entails a 20–30% loss of vitamin A and carotene (102) and there is further considerable loss on storage. The stability of vitamin A in ghee at temperatures encountered in various cooking processes is recorded in (103).

Kehar *et al.* (85) report extremely low vitamin A potencies in ghee samples purchased in bazaars in India. They attribute these to heavy losses during storage.

### (*b*) *Vitamin D*

The revised A.O.A.C. method for determining vitamin D is described in (104), and is also reported by Friedman (105). Kon & Thompson (71) find that none of the chemical methods of assay are suitable for the very low concentrations of vitamin D which occur in natural milk (not more than 0·001 $\mu$g./g.).

### (*c*) *Vitamin E*

The tocopherol content of Dutch butter has been investigated by Kruisheer (106, 107). Marked seasonal variations were found, tocopherol being lower during stall feeding periods and showing a significant correlation with carotene levels. The tocopherol in butter did not show anti-oxidant activity of any importance and there was some evidence of a greater development of oxidative rancidity in butter produced during periods of pasture feeding. Hellström & Anderson (108) also found lower tocopherol value during the winter than in the summer in Swedish butters. A seasonal trend with minimum levels in midsummer but again a close correlation between tocopherol and carotene content, is reported in New Zealand butters by McGillivray (76). Naraynan *et al.* (83)

report levels for the tocopherol content of cow and buffalo milks and show the dependence of the animal on the level of tocopherol in the ration for the secretion of the vitamin in the milk (99). As was the case with vitamin A, tocopherol levels recorded were distinctly higher during the monsoon months.

A review on the physiology, pathology and clinical importance of tocopherol (109) includes a section on tocopherol in milk and milk products. The use of the tocopherol content of the fat of dairy products as a valuable index of adulteration, has been suggested by Mahon, Aglin & Chapman (110).

*(d) Vitamin K*

The vitamin K content of milk is discussed by Rechcigl (111).

The feeding of menadione to cows at levels up to 10 g. per cow per day had no direct effect on the rate of lactic acid development in the milk (112). The direct addition of menadione to milk reduced the rate of lactic acid formation, but the levels necessary to arrest it completely over long periods (100 $\mu$g./g.) produced undesirable colour and flavour changes in the milk.

*(e) Vitamin fortification*

The fortification of milk and milk products with fat-soluble vitamins, particularly A and D, has been further investigated by a number of workers and has given rise to some sharp differences of opinion. In an excellent series of papers on the fortification of foods with vitamin A and D, Moore (113) discusses nutritional requirements for these vitamins, and Booth (114) surveys dietary supplies. The paper on the fortification of foods by Williams (115) deals mainly with margarine but refers to vitamin D in milk. Regulations governing the vitaminization of foods, including liquid and dried milk, in a number of countries are described in (116).

A method for producing skim-milk powder fortified to contain 8000 i.u. vitamin A/lb. is described by Conochie & Wilkinson (117). The loss of vitamin A was found to be less than 10% after 6 months storage at 40° C. Investigating the stability of vitamin A in fortified liquid milk and milk powders, Cox, Coulter & Lundberg (118) found that gas packing under nitrogen or the addition of the anti-oxidant, nordihydroguaiaretic acid, effectively protected the vitamin in the milk powders. NDGA also retarded destruction of vitamin A in liquid milk. Results of work on the relative stability of synthetic vitamin A in ghee and other oils on storage are given in (119).

Methods of increasing the vitamin D content of milk are reviewed by Engel (120) who concludes that milk treated by the controversial Scholl, Scheer & Steinheil process is effective in preventing and curing rickets and has a vitamin D potency of 160–200 i.u./l. as compared with an average level of 20 i.u./l. in untreated milk. Scheer (121) reports comparable vitamin D levels of 5 i.u./g. fat, obtained in a number of laboratories, in irradiated evaporated milk. Attention is drawn to drawbacks of the process of milk irradiation, such as destruction of vitamins and proteins and the possible formation of dangerous radiation by-products, by Kollmann (122) who prefers direct addition to milk of vitamin D in alcoholic solution. Holtz, Pfennigsdorf & Ponsold (123) do not, however, agree and consider that direct addition is fraught with greater dangers than irradiation.

Conflicting views are also expressed on the merits of fortifying milk with vitamin D. Weech (124) supports the idea and considers that the widespread practice of fortifying milk with vitamin D has been the most important factor responsible for the eradication of rickets. Forbes (125), however, suggests that the over-fortification of foods, including milk, with vitamin D may be contributing to the increasing incidence of hypercalcemia in infancy. While considering it undesirable to omit the added vitamin from milk, its incorporation in strictly defined amounts is advocated in (126). Pros and cons of the fortification of milk with vitamin D are also discussed by Cathala & Hennequet (128).

To test the hypothesis that the routine addition of vitamins and minerals to milk might cause adverse effects on health and well-being, feeding trials were carried out over several generations of rats by Teply, Prier & Scott (127), but no deleterous effects attributable to the supplements were observed.

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## PART 2

### INTRODUCTION

This part of the review of the nutritive value of milk and milk products is based on a selection of papers concerned with the proteins, carbohydrate, minerals, and water soluble vitamins. Hansen & Carlson (1) have reviewed present knowledge of the value of milk as a food.

Recent advances in methods of milk processing have introduced new products, and much of the recent work has been concerned with the nutritive value of milk treated by these new methods. The increasing use of very-high-temperature (VHT) sterilization, in which milk is subjected to a temperature of 135° C. for a few seconds is discussed in (2). Unfortunately as there is at present no satisfactory technique for aseptically bottling the product it must be further sterilized after bottling. A new type of dried skim-milk—instant non-fat milk—was introduced in the United States in 1954. As its name implies, it is a dried powder that can be rapidly and easily reconstituted. It is prepared by further processing of spray-dried low-heat skim-milk (3). The use of ionizing radiations for the sterilization and preservation of foods is fully discussed by Hannan (4) who considers that this technique is unlikely to have commercial application to milk because of the intensity of the flavours developed. General aspects of radiation sterilization of milk and the effect on flavour are considered in (5,6), and some of the compounds causing off-flavour which are formed during the irradiation of skim-milk and sodium caseinate are identified in (7).

The influence of foot and mouth disease on the compositional quality of milk is reviewed in (8). The use of hydrogen peroxide as a dairy preservative is discussed by Lück (9), who claims that it does not reduce the nutritive value of milk to any greater extent than other accepted methods of preservation.

## MILK PROTEINS

### *General*

Processes are described for producing a water-soluble casein through removal of the ionic calcium by a sequestrant (10), and for preparing vitamin-free casein by solution and reprecipitation from dilute phosphoric acid followed by exhaustive extraction, first with ethyl alcohol and then with ether (11).

The amino-acid composition of crystalline α-lactalbumin is given in (12); the high tryptophan content (7%) is noteworthy. The Commonwealth Bureau of Animal Nutrition has prepared, from published values, a useful collection of tables showing, *inter alia*, the amino-acid composition of milk, milk proteins and milk products (13).

The nutritive value of milk proteins is discussed in detail by Henry (14).

### *Biological value*

In an investigation of the effect of VHT treatment, Henry (15) found biological values for rats of 92, 93 and 88, respectively, for freeze-dried raw milk and for VHT sterilized milk before and after subsequent in-bottle sterilization; the last value was significantly lower than the others and confirmed the earlier finding (16) of a lower value for sterilized than for raw milk. De Groot & Engel (17) made a similar study of milk treated by the Stork two-phase process which closely resembles that used for the samples examined by Henry (15). They found biological values for rats of 87, 88 and 85 for raw, pasteurized and sterilized milk and thus confirmed that VHT treatment did not affect the nutritive value of the milk proteins; the small decrease in the biological value of sterilized milk was not significant. Growth tests with 2- to 4-day old piglets also showed no difference between the products.

Hodson (3) showed that there was no significant difference in the protein efficiency ratios for growing rats of pasteurized milk, a composite sample of spray dried low-heat skim-milk, and the same composite after reprocessing to instant milk; the values were 2·7, 2·9 and 2·9. Samples of the dried products were kept for 2 years without deterioration in moisture-proof containers. A sample in a cardboard box showed increase in moisture content from 3·3 to 5·8% and a slight loss of lysine, as determined by microbiological assay of an acid hydrolysate, but rat-growth tests indicated no loss of nutritive value (18).

Sterilization of frozen evaporated milk by a $3 \times 10^6$ r.e.p. dose of gamma irradiation was found by Metta & Johnson (19) to lower the biological value from 90 to 82, whereas heat sterilization of the same milk gave a value of 84; the true and apparent digestibilities of the sterilized products were the same. In further experiments they showed that the biological value of a sample of irradiated milk was increased from 74 to 92 by the addition of 0·3% of cystine; additions of lysine and histidine were without effect. It seemed probable, therefore, that the lowering of the biological value of milk proteins was due to either destruction or binding of cystine (20). Irradiation of evaporated milk with a $2 \times 10^6$ r.e.p. dose of gamma rays caused no change in the PDR index of net protein utilization (see below), though the amounts of cystine released by enzyme digestion was reduced by 57% (21).

The generally higher biological value for rats of spray-dried, as compared with roller-dried, skim-milk was confirmed by Richter & Schiller (22), who found values for a series of nine spray-dried powders to range from 86 to 97, whereas those for thirteen roller-dried powders were from 80 to 96; the higher values for the latter were for milk roller-dried at relatively low temperatures.

### *Studies of changes in milk proteins during heat treatment*

The changes in the biological value of milk proteins during the heat treatment of milk have their origin in changes in the content or availability of individual amino-acids, for the nutritive value of a protein depends not only on its content and balance of essential amino acids but also upon the availability of the amino-acids to the animal.

Sheffner, Eckfeldt & Spector (22) have evolved a method for calculating values for net protein

utilization that takes account of the physiological availability of amino-acids during digestion. This new procedure is essentially a modification of the amino-acid index method of calculating biological values of proteins (cf. Mitchell (23)) that frequently show good agreement with those determined with animals. Sometimes, however, the calculated values are too high, which suggests that part of the potential nutritive value of the protein has been lost during digestion. The new index, the Pepsin-digest-residue (PDR) amino-acid index combines the pattern of essential amino-acids released by *in vitro* pepsin digestion with the amino-acid pattern of the remainder of the protein. The PDR index showed good correlation with values for net protein utilization determined with rats for a number of native and heat-treated proteins. Unheated vitamin-free casein gave a PDR value of 68; after heating to 350° F. in an oven for 1 hr. the value fell to 39 and after 5 hr. to 23, the corresponding net protein utilizations were 82, 44 and 24 (24). The results for irradiation-sterilized milk (see p. 353) seem less satisfactory, and there is little doubt that *in vitro* studies of the liberation of amino-acids from proteins may not give a true index of availability in the intact animal where, as Gupta & Elvehjem (25) stress, a multiple enzyme system acts during the process of digestion, and other factors such as rates of absorption of the amino-acids and their simultaneous appearance at the sites of protein synthesis within the body influence the determination of availability.

*Lysine destruction in heated milk*

The main effect of heat on milk proteins is to reduce the availability of lysine through a Maillard reaction. The nature and extent of this loss has been investigated in several ways. Thus Schober & Prinz (26) used a method based on the reaction of lysine with dinitrofluorobenzene to show that heating skim-milk at 100–125° C. for up to 20 min. caused a progressive decrease in lysine content, but that spray and roller drying did not appreciably affect it. In other experiments they determined the concentration of lysine in the portal blood of dogs receiving unheated or heated milks, and found that 15–40% of the lysine and tyrosine (not separable by the method used) were made unavailable by sterilizing milk at 112° C. for 17 min. (27). Mauron (28) has republished the details of the investigation of the fate of lysine during milk processing (29), and included a brief account of confirmatory rat-growth tests. Interesting model experiments on the interaction of glucose and lysine peptides provide strong support for the view that the unavailability of lysine after the Maillard reaction in heat-treated milks is due to difficulty in the enzymic hydrolysis of the peptide bonds involving lysine (30, 31).

The partial unavailability of lysine from sterilized evaporated milk may be of importance in infant nutrition. The adequacy in infant feeding of the lysine content of milk proteins has been the subject of much recent discussion since Albanese, Higgons, Hyde & Orto (32) made the surprising claim that milk may be deficient in lysine. These workers chose to assess the nutritional value of milk protein in terms of its lysine/tryptophan ratio. Their determination of this ratio gave values of 3·4:1 and 4·3:1 for human and sterilized evaporated cow's milk, respectively. The infant was found to require a ratio of 6–7:1, i.e. 180–210 mg. of lysine per kg. body weight per day, based on an established tryptophan requirement of 30 mg. per kg. body weight per day. Thus they postulated that an infant receiving 3·5–4·0 g. of total milk proteins per kg. body weight per day would benefit from the addition of 100 mg. of L-lysine per kg. body weight per day. Observations on a series of infants confirmed that improved growth and nitrogen retention were obtained a in number of instances when lysine was added to the milk of undernourished infants; no effect was found with infants growing at a more than average rate.

These findings have been criticized by Hansen & Carlson (1), who rightly point out the pitfalls of assessing the value of proteins in terms of amino-acid ratios. They consider that a lysine/tryptophan ratio of 5:1 is nutritionally adequate and that the ratio in cow's milk is 5·5:1. In fairness to Albanese *et al.* it should be mentioned that their value of 4·3:1 was obtained for sterilized evaporated milk in which some destruction of lysine may have occurred. More searching criticism comes from Snyderman, Holt, Norton & Fowler (33), who found a much lower lysine requirement of 90 mg. per kg. body weight per day, and thus a required lysine/tryptophan

ratio of 3:1, which is exceeded in both human and cow's milk (even after sterilization), so that the question of the need for a lysine supplement does not arise.

The addition of lysine to a cow's milk diet did not improve nitrogen absorption or retention in five Mexican children with chronic severe protein malnutrition (34).

Further evidence for the adequacy of the lysine content of milk comes from Karte (35), who studied the changes in the physical properties of milk proteins at successive stages during the preparation of evaporated milk, and found that none of these changes affected the biological value or digestibility of the milk proteins in infant feeding.

Rice (36), reviewing the lysine content of milk proteins, considers that the case for supplementation is not yet proven and that there is, in fact, some danger of producing an amino-acid imbalance by the addition of lysine until the evidence for such addition is conclusive.

*Supplementary relationships*

The supplementary relationship between milk and cereal proteins is again illustrated by experiments in which rats showed improved growth when 3–12% of dried skim-milk was incorporated in their bread diet (37, 38, 39, 40); the addition of 0·166 or 0·25% of lysine caused a further improvement (37, 38, 40). The nutritive value of rye bread was increased more by the addition of 5% of dried whey and 2% of lactose molasses than by 7% of dried whey (41).

When, however, the proportion of milk to cereal is increased, as in the use of milk with cereals in infant feeding, where approximately 5 parts of liquid milk are added to 1 part of cereal, further addition of lysine is of no benefit. Thus the addition of lysine did not improve the growth of weanling rats receiving a diet containing 70% of mixed cereals and 30% of dried skim-milk (equivalent to 3·5 parts of liquid milk to 1 part cereal) (42).

*Miscellaneous*

Following the observation (43) that sterilization of rat diets with ethylene oxide caused losses of nutrients, Windmueller, Ackerman & Engel (44) showed that the nutritive value of casein for weanling rats is seriously impaired when it has been exposed to ethylene oxide for 24 hr. Normal growth was restored by supplements of histidine and methionine. Microbiological assays of a sample of fumigated casein showed that 71% of the histidine and 56% of the methionine originally present had become unavailable to *Lactobacillus mesenteroides*.

It is of interest that ethylene oxide did not react with dry powdered histidine or methionine; the only amino-acid that reacted was cysteine.

## WATER SOLUBLE VITAMINS

*Analytical methods and compositional data*

No important changes in assay procedures have been proposed during the last two years. Kon & Thompson (45) have described, with full experimental details, the methods used at the National Institute for Research in Dairying for the physicochemical and chemical measurement of thiamine, riboflavin and ascorbic acid. Hodson (46) discussed modifications of the microbiological assays for vitamin $B_6$ with *Neurospora sitophila* 299 and *Saccharomyces carlsbergensis* 4228, and concluded that his earlier results were too high (see p. 357). The pantothenic acid contents of milk and milk products, as determined by microbiological assay with *Lactobacillus plantarium*, are given in (47).

In a comprehensive study of the effect of processing Chapman, Ford, Kon, Thompson, Rowland, Crossley & Rothwell (48) determined thiamine, riboflavin, nicotinic acid, pantothenic acid, biotin, vitamin $B_6$ and vitamin $B_{12}$ in raw, pasteurized, sterilized, VHT-treated, VHT-treated and sterilized, evaporated, sweetened condensed, and roller- and spray-dried milks prepared simultaneously from a bulk supply of raw whole milk. There was little or no apparent loss of riboflavin, pantothenic acid, nicotinic acid, vitamin $B_6$ or biotin. Thiamine losses were less than 10% during pasteurization, VHT treatment or spray drying, 30–40% during in-can sterilization of

bottled milk and 40–50% during in-bottle sterilization. Cyanocobalamin was almost completely destroyed during sterilization (see p. 358), and losses of 30 and 40% occurred during roller- and spray-drying and condensing, respectively; pasteurization caused a 10% and VHT treatment a 15% loss. Hartman, Dryden, Moore & Hodgson(49) reported similar losses of vitamin $B_{12}$ during condensing and sterilizing. The effect of stage of lactation on the concentrations of biotin, nicotinic acid, pantothenic acid, riboflavin and vitamins $B_6$ and $B_{12}$ in the milk of Shorthorn and Friesian cows was studied in (50). Considerable variations were found on the levels of the vitamins, both between individual cows and from day to day in the same cow; these variations were particularly marked for biotin and vitamin $B_{12}$. The mean levels of biotin, pantothenic acid and vitamin $B_6$ rose to peaks between the fourth and seventh day after calving, and thereafter fell to the normal milk level. The mean level of nicotinic acid fell slightly and then recovered during the first week and then fell gradually up to the fifth month; it rose again thereafter, probably as an effect of season. As expected the concentrations of riboflavin and vitamin $B_{12}$ were higher in colostrum than in later milk. The only significant breed difference found was that the level of riboflavin was 30% higher in Friesian than in Shorthorn milk; this difference may have been accentuated because the levels in the Shorthorn milk were unusually low.

Some other recent publications listing determinations of vitamins in milk and milk products are summarized in the following paragraphs.

Values from Poland for the thiamine and riboflavin content of raw and boiled milk (boiling caused reductions of 7 and 2%, respectively) are given in (51), and for riboflavin in milk (52). Data for the riboflavin and thiamine content of milk in Russia during a 12-month period are given in (53). Italian workers found, rather surprisingly, that the riboflavin content of milk was markedly reduced when the cows were given acid silage (54). The thiamine, riboflavin, nicotinic acid and ascorbic acid contents of mare's milk and koumiss, and cow's milk are compared in (55). Cows suffering from foot-and-mouth disease secreted mik containing 25% less riboflavin, but 20% more thiamine and 60% more ascorbic acid (56). The vitamin $B_{12}$ content of bulk cow's milk was 3·9 $\mu$g./l. in Finland (57,58) and 5·35 $\mu$g./l. in Russia (59).

Anagama (60) has published a full account of his extensive study of reduced ascorbic acid in milk and milk products in Japan. Slightly higher mean values were found for milk from Guernseys (23·4 mg./100 ml.) than from Holsteins (16·4 mg./100 ml.). No consistent effects were found of feed, season or stage of lactation. Processing caused the expected losses, except for the very remarkable finding that HTST pasteurization caused a more than 50% loss, whereas holder pasteurization caused a loss of about 10%.

Seasonal and farm-to-farm variations in the ascorbic acid content of milk are claimed in (61), but no effect of season or stage of lactation was found in (62). The enrichment of cow's milk with ascorbic acid was studied in (63). Values are given for the ascorbic acid content of cow's milk in Poland (64, 65) and of dried milk in Argentina (66).

A reduced loss of ascorbic acid from milk exposed to light in cartons instead of glass bottles was confirmed in (67).

The choline contents (mg./100 ml.) of the colostrum and milk respectively of various species were: cow, 48–72 and 6–15; goat, 35–143 and 15; ewe, 77–103 and 5; mare, 36–60 and 3–4; sow, 30–90 and 0–28 (68); values of 9 and 40 for cow's milk in winter and summer are given in (62).

A sample of musk-ox milk contained 3·4 $\mu$g. of vitamin $B_{12}$ per litre (69).

Mean values for the ascorbic acid content of fresh and soured ewe's milk and fresh and soured buffalo's milk in Bulgaria were 3·3, 2·2, 2·3 and 1·4 mg./100 ml., respectively (70).

The level of pantothenic acid in human milk ranged from 1·98 to 3·55 $\mu$g./ml. and was slightly lower in colostrum (71). The thiamine content of the milk of Russian mothers was 3–33 $\mu$g./ml., depending on the dietary thiamine intake; administration of 20–30 mg. of thiamine increased the level in milk to 26–47 $\mu$g./ml. but smaller doses were ineffective (72). The cyanocobalamin content of human colostrum was 300 m$\mu$g./100 ml., declining with advancing lactation to 20–30 m$\mu$g./100 ml. after 2–3 months; the level in milk could be increased by intramuscular injection of cyanocobalamin more easily than by oral administration (73).

The conversion of low heat dried skim-milk to instant milk had no measurable effect on the content of thiamine, riboflavin, nicotinic acid, pantothenic acid or vitamin $B_6$ (3). Irradiation sterilization of dried milk was without effect on the thiamine, riboflavin and nicotinic acid content (74).

Cheese retains about 40% of the pantothenic acid originally present in the milk (47). The pantothenic acid content of 306 samples of twenty-one cheeses ranged from 211 to 3954 $\mu$g./100 g. (75). The vitamin $B_{12}$ potency of Cheddar, Swiss and Cottage cheeses were studied by Hartman, Dryden & Hargrove (76), who compared results from microbiological (*Lactobacillus leichmannii*) and rat assays; the results from the latter were generally almost twice those determined microbiologically.

Small losses of thiamine and riboflavin during the manufacture of yoghurt were reported (77). The riboflavin content of buffalo milk curds was 1–1·7 $\mu$g./g. (78).

*Riboflavin*

The form in which riboflavin occurs in milk is known to vary with species (79), for instance cow's milk was found to contain mainly free riboflavin, whereas sow's milk contains principally flavinadenine dinucleotide (FAD) (80). Recently the partition of riboflavin between free riboflavin, riboflavin-5′-phosphate and FAD in the milk of several species was further studied by Modi & Owen (81), who found that the milk of cows, goats, ewes and rabbits contained mainly free riboflavin whereas human, mare's and sow's milks contained only FAD. In a subsequent paper Manson & Modi (82) investigated the occurrence and metabolism of FAD in the milk of the sow and cow. The free riboflavin in cow's milk and a proportion of the FAD in sow's milk were ultrafiltrable from unheated milk, but heating the milks at 95° C. for 3 min. denatured the milk proteins and liberated all the FAD in both milks. When FAD was added to fresh cow's milk it was rapidly destroyed with the formation of free riboflavin and riboflavin-5′-phosphate, but when it was added to pasteurized milk that showed no phosphatase activity the final product was riboflavin-5′-phosphate. There was no alteration of FAD added to sow's milk. In cow's milk some of the FAD is present as xanthine oxidase, but no xanthine oxidase activity could be demonstrated in sow's milk and it was suggested that sow's milk must contain a different combined form of FAD.

The level of xanthine oxidase activity in milk in relation to the molybdenum intake of the cow was studied in (83,84). The mean activity of the milk of cows receiving fodder containing 13 $\mu$g. of molybdenum/100 g. was only about one-tenth of that of the milk of cows grazing grass containing 353 $\mu$g. of molybdenum/100 g. dry matter. Preliminary findings indicate, however, that an inorganic molybdenum supplement does not cause such a marked increase.

*Vitamin $B_6$*

It is becoming evident that cases of convulsive seizure in infants due to an insufficient intake of vitamin $B_6$ from commercial milk preparations (cf. (85)) are probably due to an abnormally high requirement for this vitamin by a small minority of infants (86). None the less, inquiry continues into the stability of vitamin $B_6$ in milk to processing. Such studies are seriously hindered by the lack of really satisfactory differential assays for the three components of vitamin $B_6$ -pyridoxine, pyridoxal and pyridoxamine. Hodson (46) has conducted a careful appraisal of the available microbiological assays. Using assays with *Neurospora sitophila* 299 and *Saccharomyces carlsbergensis* 4228, organisms responding to all three components, and with *Lactobacillus casei*, which responds only to pyridoxal, he investigated the behaviour of vitamin $B_6$ in milk during sterilization and subsequent storage of the product. He concluded from these studies that, whereas nearly all the vitamin $B_6$ of fresh and of non-sterilized evaporated milk is in the form of pyridoxal, after sterilizing only part of the activity was due to pyridoxal, the remainder was probably pyridoxamine formed from pyridoxal by reaction with amino groups. The activity of stored sterilized evaporated milk was not due to either pyridoxal or pyridoxamine, and he postulated that it was present as yet another form of vitamin $B_6$ that has activity equal to

pyridoxine for *Neurospora*, but much less for *Saccharomyces carlsbergensis* and still less for the rat. Its activity for *Lactobacillus casei* compared to a pyridoxal standard was about the same as its activity for *Saccharomyces carlsbergensis*.

*Vitamin $B_{12}$*

The almost complete destruction of cyanocobalamin during sterilization of milk has already been mentioned (p. 356). Ford (87) has made a detailed study of factors influencing the destruction by heat of vitamin $B_{12}$ in milk. The process appeared to be oxidative and could be prevented by adding sodium cyanide or potassium metabisulphite to the milk before heating. Other reducing agents, such as reduced glutathione, thioglycollic acid and ascorbic acid, enhanced the destruction. The mechanisms underlying these effects are not yet fully understood. The most effective practical means of minimizing the loss of vitamin B was deaeration of the milk before heating; this procedure also conserved ascorbic acid.

## MINERALS

The human requirements for calcium are reviewed by Widdowson (88), who considers dietary factors influencing the absorption of calcium, and discusses the causes of hypercalcaemia in infants fed on cow's milk. The pathogenesis of idiopathic hypercalcaemia is further discussed in (89).

In studies with $^{45}Ca$, Wasserman, Comar & Nold (90) confirmed that lactose improves calcium utilization and showed that certain amino-acids had a similar though smaller effect. Thus the amount of $^{45}Ca$ in the femurs of rats given a single oral dose of $^{45}Ca$ was almost doubled when a small amount of lysine was given with the calcium (91). In other experiments these workers studied the absorption of $^{45}Ca$ and $^{89}Sr$ from milk and non-milk diets given to cattle, rats, rabbits and man and showed that the retention of single oral doses of $^{45}Ca$ and $^{89}Sr$ gave essentially the same results as determinations of calcium balance. They found that the retention of calcium by cattle and rats was 50% higher when a dose of $^{45}Ca$ and calcium chloride was given with milk than when it was given alone or with a grain mixture; there was no such difference with rabbits. Strontium absorption from milk by three out of four human subjects was more than twice that from an aqueous solution of calcium chloride (92).

These studies comparing the utilization of calcium and of strontium are of topical interest in relation to the increase in the $^{90}Sr$ content of milk due to contamination of pastures and herbage with the fall-out of radioactivity from nuclear weapon tests. The fission products formed in nuclear explosions are carried round the world in the upper air and may take 10 years to fall to earth. General problems concerned with the inter-relationships of strontium and calcium in the biological cycle, from soil to plants to animals and back to soil, are discussed in (93, 94, 95).

Although the metabolism of strontium closely resembles that of calcium, strontium is less efficiently absorbed and preferentially excreted, and the ratio of strontium to calcium in cow's milk is about one-tenth of that in the rations fed. None the less, since milk is the principal source of calcium in the diet of young children, it is important to follow any changes in the $^{90}Sr$ content of milk. The results of measurements at Harwell (94, 95) of $^{90}Sr$ in milk and in the bones of young children during 1955–57 show that although there were small increases the concentrations are still very far below the accepted tolerance levels.

## LACTOSE

The manufacture of lactose, its chemical and physical properties and its uses, are described in (96). The physiological effects of lactose were reviewed by Duncan (97), and its use in animal and human feeding by Atkinson, Kratzer & Stewart (98).

It has been known for many years that the inclusion of high levels of lactose in the post-suckling diet of many animals resulted in poor growth and frequently caused diarrhoea, and that

equivalent amounts of glucose and galactose did not have these effects. Further similar findings with rats are reported in (99, 100, 101, 102).

The problems associated with the utilization of lactose are not fully elucidated, but interest in its role in nutrition centres on certain characteristic effects not shared by other sugars. Some of these are due to galactose liberated on hydrolysis, and others are specific to the lactose molecule itself, and derive from its less rapid efficient digestion than that of other disaccharides. Lactose, in common with other disaccharides, is normally not absorbed intact, but must be broken down by the specific enzyme lactase to glucose and galactose before passage into the blood stream and utilization in the body. It is evident from recent studies that in many species lactase activity is highest during the first few days of life and that it declines rapidly thereafter. In the rat (103, 104) and the pig (103, 104, 105, 106), for instance, the lactase activity of the intestinal mucosa has fallen by weaning to about one-tenth of its initial value. Contrariwise, the activities of sucrase and maltase increase during early life. Thus, whereas the suckling animal can utilize lactose, growing and adult animals are less well equipped to utilize a diet high in lactose than one high in sucrose or starch-degradation products.

In a rare human disease, congenital galactosaemia, infants are unable to metabolize galactose. Milk feeding in galactosaemic infants leads to increase in size of liver and spleen, ascites, cataract and feeble mindedness (107, 108). These symptoms rapidly disappear when milk is replaced by a galactose-free diet. Galactose is normally metabolized after conversion to glucose; the series of reactions involved has recently been elucidated by Kalckar and his co-workers and is summarized in (109). Briefly the steps are:

$$(1)\quad \alpha\text{-D-galactose} + \mathrm{ATP} \xrightleftharpoons[\text{kinase}]{\text{galactose}} \alpha\text{-D-galactose-I-P} + \mathrm{ADP}$$

$$(2)\quad \alpha\text{-D-galactose-I-P} + \underset{\substack{\text{uridine}\\ \text{diphosphoglucose}}}{\mathrm{UDPG}} \xrightleftharpoons[\text{uridyl transferase}]{\text{galactose-I-phosphate}} \text{glucose-I-phosphate} + \mathrm{UDPGal}.$$

$$(3)\quad \mathrm{UDPGal} \xrightleftharpoons[\substack{\text{diphosphogalactose}\\ \text{4-epimerase}}]{\text{uridine}} \mathrm{UDPG}$$

The biochemical lesion in galactosaemia is a lack of galactose-I-phosphate uridyl transferase (110). The condition can be detected by studying the galactose metabolism of red blood cells taken from cord blood at birth (111). If impaired metabolism is found the child must be given a lactose- and galactose-free diet.

### MILK AND MILK PRODUCTS FOR THE PRE-RUMINANT CALF

The increasing adoption of the early weaning technique developed by Preston (112, 113, 114), in which calves are weaned from a liquid to a solid diet abruptly and completely at 3 weeks of age means that milk substitute diets have a more limited application than heretofore. This is particularly so in the rearing of animals as herd replacements, for the system requires the feeding of only about 10 gallons of whole milk to each calf.

There is still a need, however, for a satisfactory substitute for cow's milk in the rearing of calves for veal or beef, especially for the non-dairy farmer who may wish to buy in and rear calves, and interest continues in the study of the nutritive requirements and digestive capabilities of the young calf.

Carbohydrate utilization in the young calf was studied by Dollar & Porter (115), who found that during the first 4 weeks of life the non-ruminant calf utilized only glucose and lactose of the carbohydrates tested (glucose, lactose, maltose, sucrose, dextrin and starch). At 9 weeks there was some utilization of maltose. These findings correlated with the measured activities of

lactase, maltase, sucrase and amylase in the intestinal mucosa and pancreas of the calf. Thus lactase activity was high at birth, whereas maltase and amylase activities were negligible at birth and increased only very slightly thereafter; no sucrase activity was detected even at 5 weeks. Dollar & Porter concluded from these results that cereal flours, either raw, dextrinized or cooked, serve no useful purpose as sources of energy for calves in the pre-ruminant period of life.

There are several reports showing that the calf can utilize a wide range of dietary fats, provided they are suitably emulsified; results with saturated fats are generally superior to those with unsaturated ones (116).

Present knowledge of protein utilization in the calf is scanty. Milk proteins are well utilized, but vegetable proteins have proved less satisfactory with young calves. Thus maize and soya proteins were poorly used from a milk replacement diet until the calves were 4 weeks old (117, 118), but in other experiments (119,120) reasonable growth was obtained during the first 4 weeks of life when α-protein (an isolated soya protein) was the sole protein source in a synthetic diet. However, until the processes involved in protein digestion in the calf are more fully understood it is probable that milk proteins will remain the proteins of choice in milk substitute diets.

Preston (121) discusses the formulation of milk substitute diets from dairy products including buttermilk, dried skim-milk and dried whey. He reports satisfactory results with reconstituted roller-dried buttermilk and with reconstituted roller-dried skim-milk. Dried buttermilk has also been used successfully in New Zealand (122,123).

### MILK PRODUCTS FOR THE PIG

Milk products have two important applications in pig feeding: they are important ingredients in the diet of artificially reared pigs, especially during the first 2 weeks of life, and products such as skim-milk, whey or buttermilk are fed to pigs as a convenient and useful means of utilizing these nutritionally valuable products. Especially since the increasing production of cheese and butter in Great Britain is resulting in the preparation of more skim-milk and whey than the available drying plant can handle, particularly during the late spring and early summer.

Problems associated with the choice of suitable diets for artificially reared and early-weaned pigs are discussed in reviews by Bellis (124), Braude (125) and Catron (126,127). Present evidence shows that milk proteins are the most satisfactory source of protein during at least the first 2 weeks of life. The only carbohydrates the new-born pig can utilize are glucose and lactose (128), but the enzymes necessary for the digestion of sucrose and maltose develop within the first week of life, and those for the digestion of starch by 14 days (106, 129, 130).

The uses of liquid skim-milk, either fresh or soured, and dried skim-milk as protein supplements to the diet of fattening pigs requires little elaboration. Braude (131) reviews the advantages of skim-milk as a food for pigs, and discusses the merits of the addition to skim-milk of formalin as a preservative. Barber, Braude & Mitchell (132) found, in preliminary trials, that the addition to skim-milk of 0·1–0·5% of formalin (40% solution of formaldehyde) enabled it to be stored for several weeks without clotting. The milk so treated could be fed satisfactorily to fattening pigs and the bacon produced from them had a normal flavour.

Whey is used in pig diets primarily as a source of energy. About 12% of its dry matter is in the form of protein, about 9% as minerals, and the remainder is lactose. Thus problems associated with the use of high levels of dried whey are essentially those of lactose utilization, though there are suggestions that the high mineral intake may also be detrimental. Studies by Becker, Terrill, Jensen & Hanson (133) showed that up to 60% of dried whey could be included in a semi-synthetic diet for suckling pigs, but weanling pigs could tolerate only 20–30% in a practical diet; heavier pigs could tolerate higher levels in a semisynthetic diet, but not more than 20% in a practical diet. It appeared, therefore, that older pigs had a reduced capacity for utilizing lactose, though it was noteworthy that the composition of the remainder of the ration had a marked effect on the amount of whey that could be fed. Similar findings are reported by Dunkin (134) in a brief summary of his investigations of the physiological effects of feeding high levels of whey.

When liquid whey is used the problems are greatly simplified because the lactose intake is regulated by the animal's capacity for liquid. Thus fattening pigs given 2 lb. of meal per day and offered whey *ad lib.* drank approximately 4 gallons each day (135), so that the level of lactose in their diet restricted itself to about 30%.

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